MICROBIAL DEGRADATION OF THE FUEL OXYGENATE METHYL *TERT*-BUTYL ETHER (MTBE)

by

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A Dissertation submitted to the

Graduate School of New Brunswick

Rutgers, The State University of New Jersey and

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Microbiology and Molecular Genetics

Written under the direction of

Dr. Max M. Häggblom

And approved by

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New Brunswick, New Jersey

October, 2009

ABSTRACT OF THE DISSERTATION

Microbial degradation of the fuel oxygenate methyl *tert*-butyl ether (MTBE)

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Dissertation Director:
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Groundwater contamination with the synthetic fuel oxygenate, methyl *tert*-butyl ether (MTBE), is an extensive problem. Microbial mediated biodegradation holds promise as a tool for remediation of contaminated water supplies. However, MTBE biotransformation processes are slow and MTBE degrading organisms are difficult to isolate, creating challenges relating to site assessment, enhancement of natural attenuation and monitoring bioremediation *in situ*. In this study we analyzed MTBE degrading cultures using a variety of isolation independent techniques. A majority of the experiments used previously established anaerobic enrichment cultures that had been maintained on MTBE for several years. We demonstrated that low concentrations of some aryl O-methyl ether compounds enhanced the rate of MTBE degradation. Propyl iodide caused a light-reversible inhibition of MTBE depletion, suggesting that the anaerobic MTBE O-demethylation reaction was corrinoid dependent. Terminal-restriction fragment length polymorphism (T-RFLP) and sequence analysis of 16S rRNA genes from one anaerobic MTBE degrading enrichment culture showed a phylogenetically diverse population with no exact matches to previously isolated or described species. Stable isotope probing experiments verified that microorganisms from anaerobic MTBE degrading enrichment culture used ^{13}C from ^{13}C-MTBE for growth and

cell division and that a particular subpopulation assimilated this carbon prior to the rest of the population. We also analyzed carbon and hydrogen stable isotope fractionation occurring during MTBE degradation. In anaerobic cultures, substantial fractionation of hydrogen was found only in cultures supplied with syringic acid during MTBE degradation, providing the first experimental suggestion of multiple anaerobic MTBE O-demethylation mechanisms. During aerobic MTBE degradation by the psychrophilic bacterium, *Variovorax paradoxus*, carbon and hydrogen fractionation were not influenced by incubation temperature during degradation. This work represents a significant contribution to the current body of knowledge about MTBE degradation and the data presented will be useful in many aspects of studying, enhancing and monitoring MTBE degradation under a variety of conditions.

DEDICATION

This dissertation is dedicated to my girls, Eloise & Claudette, who have made the last year of this project so interesting.

ACKNOWLEDGEMENTS

Many, many thanks are due to the following:

to my wonderful advisor, Max Haggblom, for his cheerful advisement, meticulous editing and unwavering enthusiasm for this project;

to my committee members, Tamar Barkay, Donna Fennell and Lee Kerkhof, for their kind support and suggestions;

to the first student on this project, Piyapawn Somsamak, for warnings and advice;

to Lora McGuinness for day-brighteningly friendly help with the molecular work;

to Gennadi Zaitsev for the aerobic cultures, Hans Richnow for collaboration on the CSIA work and Monica Rosell for sample analysis (and detailed spreadsheets);

to Theodore Chase for taking me on as a teaching assistant and to Emilia Rus and Allen Smith, who made the job much easier;

to Alan Antoine and Stanley Katz for giving me my start in the microbiology lab as an undergraduate and reminding me that there was more to college than horrible exams;

to Milo Aukerman for helping put things into perspective;

and finally, to my parents, Stephen Youngster and Suzanne Gibson, my sister, Tracy Youngster, and my husband, Frank Frohlich, for helping with everything, above and beyond what any graduate student could expect from their family.

This study was funded in part by the New Jersey Department of Environmental Protection, the New Jersey Water Resources Research Institute and a Transatlantic Research Fellowship from the EC-US Task Force on Biotechnology Research.

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Most Things Biodegrade Easier,
A brief introduction to MTBE

I. Background

Methyl *tert*-butyl ether (MTBE) is a synthetic chemical which is added to gasoline as an oxygenate to reduce carbon monoxide emissions and formation of ozone. Since the passage of the Clean Air Act, which mandates the use of fuel oxygenates, MTBE has been used extensively and, consequently, has been detected in groundwater as well as surface water across the United States (Squillace *et al*., 1996). Common sources of MTBE contamination in water resources include fuel spills, leaking underground storage tanks and pipelines, storm runoff, precipitation, and motorized watercrafts (Reuter *et al*., 1998; Brown *et al*., 2000). Studies of the potential health hazards have been inconclusive, but the US EPA currently lists MTBE as a possible human carcinogen. The concentration allowed in drinking water is also held to a low level due to the chemical's easily detectable unpleasant taste and odor.

There are several physical and chemical properties of MTBE that make environmental contamination a challenging problem. Relative to other gasoline additives, MTBE has a higher water solubility and a lower tendency to partition to organic matter in soil or to the vapor phase (Squillace *et al*., 1997). Thus, when MTBE is spilled, it is likely to dissolve in water and migrate quickly throughout the water system without hindrance by volatilization or adherence to soil. Less likely than other gasoline components to exit the water system due to physical processes, MTBE is unfortunately also less prone to biodegradation. Its structure includes a very stable ether bond and a bulky, quaternary carbon structure which greatly increases its resistance to degradation. MTBE was initially thought to be entirely insusceptible to microbial attack , but now is known to be degraded by only a few cultures of microorganisms, most of them aerobic.

MTBE biodegradation under aerobic conditions have been studied extensively (see reviews by Deeb *et al*. 2000; Stocking *et al*. 2000; Wilson, 2003; Schmidt *et al*., 2004; Häggblom *et al*., 2007). Aerobic MTBE degrading organisms have been isolated and characterized and are being used for assisted bioremediation systems. Further studies of different types of aerobic MTBE degrading organisms and the process will improve these technologies. Anaerobic MTBE degradation is a less well understood process. While anaerobic MTBE degradation does occur *in situ* and under laboratory conditions (Häggblom *et al*., 2007), the responsible organisms and mechanisms are unknown. Since many MTBE contaminated sites are subsurface in anoxic environments where aerobic biodegradation is impossible and elimination by physical and chemical processes are ineffective, study of the anaerobic MTBE biodegradation process is crucial if we are to eliminate MTBE contamination in the environment.

A. History of MTBE use

Oil companies began studying ether compounds as early as the 1920s for prospective use as gasoline additives. The first commercial addition of MTBE to gasoline occurred in Italy in 1973. In 1979 MTBE was approved in the United States for addition to gasoline at 1-8% by volume as an octane enhancer to replace *tetra*-ethyl lead. MTBE also works as a fuel oxygenate, increasing the oxygen content of fuel and promoting more complete burning and reducing ozone formation and carbon monoxide and hydrocarbon emissions (Kirchstetter *et al*., 1999). In the 1990s, MTBE use and production increased dramatically following the passage of the 1990 Clear Air Act

Amendments (Franklin *et al*., 2000) which mandated fuel oxygenate use in many parts of the U.S. that were suffering from severe air pollution.

The first stage of implementation was the 1992 winter oxygenated fuel program which required that during the winter months in 40 urban U. S. areas fuel consisted of 2.7% oxygen by weight. The laws did not specify which type of oxygenate had to be used; this decision was left up to the oil companies. Initially ethanol was a more popular oxygenate, but MTBE grew in popularity for financial reasons. MTBE is less expensive than ethanol and easier to manufacture (Shelly and Fouhy, 1994). It is also less volatile and therefore better for meeting emissions standards. The ethanol phase separates from gasoline, thus requiring separate transportation and storage and mixing with gasoline at the filling station. MTBE can be easily blended with gasoline at the refinery and then distributed, saving money in transportation and storage. MTBE is also less expensive to manufacture than other ether compounds, such as *tert*-amyl methyl ether (TAME) and ethyl *tert*-butyl ether (ETBE), which could also be used as oxygenates.

Increased pressure to use oxygenated fuel and the heavy preference for MTBE as the oxygenate led to drastic increases in MTBE manufacturing. In 1995, 21 billion kg of MTBE was produced in the US, the 2nd highest volume production of any synthetic organic chemical (US EPA, 1999). To meet the 1992 winter oxygenate requirements, gasoline had to contain MTBE at 15% by volume (Moyer, 2003). The next phase of implementation came about in 1995, when it became mandatory to use reformulated gasoline (RFG) containing 2% oxygen (equal to 11% MTBE by volume) year-round in 28 industrial areas. At this time, 87% of oxygenated fuel contained MTBE instead of ethanol and up to 30% of the fuel in the United States was reformulated to contain up to

15% MTBE by volume. In 1996, the state of California implemented the California Air Resources Board Phase 2, requiring RFG to be used year wide throughout the state. RFG had the additional requirements of not containing heavy metals and limiting benzene content to 1% (Stern and Kneiss, 1997).

In 2003, production of MTBE began to decline as use of the chemical in gasoline was banned or restricted in many states. Frequent reports of widespread groundwater contamination led the Report of the Blue Ribbon Panel on Oxygenates in Gasoline, by the US Environmental Protection Agency, to conclude that MTBE use needed to be decreased (US EPA, 1999) Other countries came to the same conclusion. In most cases, in the U. S. MTBE is being replaced by ethanol and in Europe with the other ether compound oxygenates, ETBE and TAME (Häggblom *et al*., 2007). In the absence of use as a fuel additive, demand for MTBE is small as its use is largely restricted to medical, for dissolving gallstones (Johnston and Kaplan, 1993), and laboratories, as an extraction solvent.

B. Properties of MTBE

MTBE ($C_5H_{12}O$; m.w. 88.15) is a 5-carbon compound with a tertiary carbon structure and ether bond. (Figure 1.1.). The physical and chemical properties of MTBE make environmental contamination a challenging problem (Squillace *et al*., 1997). Most treatment plans for handling gasoline spills are optimized for removing BTEX components (benzene, toluene, ethylbenzene, or o-, m-, p-xylene). Table 1.1. shows a comparison of properties of MTBE and BTEX compounds. Relative to these other gasoline additives, MTBE has a higher vapor pressure and will volatilize easily from the

non-aqueous phase. This causes greater atmospheric concentrations and distribution by precipitation. MTBE has a higher vapor density than air, leading to a tendency for MTBE vapor to sink close to land and accumulate in low areas. The solubility of MTBE in water is 50,000 mg/L, much higher than the 100-2,000 mg/L solubilities of BTEX compounds (Rosell *et al*., 2006). MTBE also has a lower Henry's law constant (ratio of concentration in air to concentration in water) than BTEX compounds, indicating a weaker tendency to volatilize from the aqueous phase. This property is more relevant to the situation of contaminated groundwater than the vapor pressure and makes MTBE more resistant to removal from groundwater by air sparging. Finally, MTBE has a lower soil adsorption coefficient (K_{oc}) than the BTEX components. This is a measure of the tendency of a compound to adhere to soil, taking into account the amount of organic carbon in the soil. MTBE's low K_{oc} causes it to be minimally retarded by soil and less susceptible to removal by frequently used carbon-based adsorption methods. Together these properties mean that when MTBE is spilled it is likely to dissolve in water and migrate quickly throughout the water system without being hindered by volatilization or adherence to soil. It is also difficult and expensive to remove by methods used for the treatment of other gasoline components.

C. MTBE, history of contamination

Throughout the 1990s and early 2000s, production, transportation and storage of a tremendous volume of MTBE led to widespread groundwater contamination, which occurred in a variety of ways during manufacturing, transport, storage and use. Spills occur during fuel transportation, storage tank filling, vehicle gas tank filling, repair and

maintenance of vehicles and other gasoline-powered equipment, and during motor vehicle accidents (Moyer, 2003). Following a spill, storm runoff can carry MTBE into the water. A study by Poulopoulos and Philippopoulos (2000) shows that fuel containing MTBE produces significant MTBE emissions during engine start-up and anytime the vehicle engine is operating at a lower power level. Volatilized MTBE has been detected at high levels in the atmosphere in some urban areas where its use was most common and could be indirectly introduced into water through precipitation. MTBE can also leak directly into surface or ground water from underground storage tanks and pipelines and from motorized watercrafts (Moyer, 2003; Gabele *et al*., 2000).

As MTBE use increased in the mid-90s, the frequency and extent of contamination was quickly visible across the country. MTBE has been detected in private wells sampled in the New Jersey area, especially wells that are near gasoline stations and other uses of gasoline (NJDEP, 2000). A USGS survey of public water supplies in 1993-1994 found MTBE to be the second most common aquifer contaminant in urban United States areas and concentrations of up to 200,000 μg/L were reported in groundwater near direct fuel leaks (Zogorski *et al*., 1997). A survey by the Northeast States for Coordinated Air Use Management group, summarized reported incidents of MTBE occurrence in 8 northeastern states and found that BTEX compounds were only detected at 12% of the sites where MTBE was found, indicative of the difference in properties between MTBE and BTEX compounds (Thomson *et al*., 2003). In 1996, soon after California decided to use reformulated gasoline throughout the state, contamination of wells in Santa Monica was discovered at levels of up to 600 ppb MTBE (US EPA, 2000). Several municipal water supplies were closed due to MTBE contamination. Despite declining MTBE use,

aquifer contamination with MTBE continues to be discovered. Studies in New Hampshire have shown that as they dig wells deeper in hopes of increasing the water yield, they are finding more contamination in the deep bedrock wells than in shallower ones (Ayotte *et al*., 2005). They have also observed greater MTBE contamination in older wells than in newer ones (Ayotte *et al*., 2008), indicating a likelihood that newly contaminated wells may continue to arise, despite the decline in MTBE use.

D. MTBE, health and environmental impact

Current limits for MTBE in drinking water are based on its organoleptic properties. MTBE has a very strong objectionable taste and smell, often compared to turpentine or rubbing alcohol, and can only be tolerated in drinking water at very low levels. Studies have reported a wide variation in responses to MTBE at different concentrations, identifying a taste and odor threshold somewhere in the range of 15 to 180 μg/L (US EPA, 1997). While there is no federal regulation regarding MTBE allowance in water, the US EPA issued a recommended limit of 20-35 ppb in drinking water (US EPA, 1997). In the interest of preserving drinking water quality, many states have adopted lower thresholds of 13-14 ppb (Ayotte *et al*., 2005). In addition to the unpleasant odor and taste, MTBE is a skin and respiratory irritant. Joseph and Weiner (2002) reported significantly higher than normal incidences of respiratory complaints in Philadelphia, PA between 1995 and 1997, when MTBE use was at its peak. Another study found a statistically significant correlation between MTBE levels in blood and symptoms of headache, eye irritation and burning of the nose and throat (White *et al*.,

1995). Bodenstein and Duffy (1998) reported that MTBE exposure causes nasal epithelial cells to express the stress protein, Hsp60, indicating cellular injury.

The US EPA currently lists MTBE as a possible human carcinogen based on animal exposure studies (US EPA, 1997; Belpoggi *et al*., 1995; Bird *et al*., 1997; McKee *et al*., 1997). Moser *et al*. (1996) reported that MTBE exposure was associated with liver tumor formation and decreased uterine weight in female mice suggesting that the carcinogenicity may be due to endocrine effects. A study by Williams-Hill *et al*. (1999) reported that MTBE induces a mutagenic pathway which may be responsible for the carcinogenicity found in some studies. More recently Caldwell *et al*. (2008) reported that tumor development in rats is directly related to MTBE exposure. In addition to carcinogenic effects, there is some evidence that MTBE exposure may cause genotoxic effects in human lymphocytes (Chen *et al*.,2008), DNA damage in mouse fibroblasts (Iavicoli *et al*., 2002), reproductive toxicity in male rats (Li *et al*., 2008) and cytotoxic effects in rabbit tracheal epithelial cells (Wang *et al*. (2008).

MTBE contamination of water supplies may also have ecotoxicological effects. Studies of the toxicity of MTBE to fish have shown toxicological effects on catfish larvae at high concentrations (Moreels *et al*., 2006a) and reproductive effects in zebrafish at levels that are often found in the environment (Moreels *et al*., 2006b). Although MTBE was not directly toxic to fathead minnow larvae, the compound increased the toxic effect of fluoranthene (Eun-ah *et al*., 2003). It has also been observed that MTBE increases the toxic effects of toluene to the Asian earthworm, *Perionyx excavates* (An and Lee, 2008), suggesting that MTBE might also increase the toxic effects of other pollutants. A study by Vosahlikova *et al*. (2006) demonstrated acute toxicity to the plant *Lactuca sativa* at

concentrations found in soil of contaminated environments. MTBE also may have an effect on some bacterial species, as Bartos *et al*. (2008) has recently shown growth inhibition of the bacterium *Pseudomonas veronii* T1/1 strain at high concentrations of MTBE exposure.

E. Studies of aerobic MTBE biodegradation

MTBE is less prone to biodegradation than BTEX compounds. The bulky, tertiary carbon structure and the high dissociation energy of the ether bond (approximately 360 kJ/mol) (Kim and Engesser, 2004) both increase the resistance of the compound to chemical and biological degradation. MTBE was initially thought to be entirely insusceptible to microbial attack, however MTBE biodegradation is now known to occur under both aerobic and anaerobic conditions. The first report of aerobic MTBE biodegradation was in 1994 (Salanitro *et al*., 1994) and since then there have been many studies demonstrating aerobic biodegradation. Aerobic MTBE-degrading cultures have been investigated and several bacteria have been identified as being able to degrade MTBE (See reviews by Deeb *et al*., 2000; Stocking *et al*., 2000; Fayolle *et al*., 2001; Fiorenza and Rifai 2003; Ferreira *et al*., 2006; Häggblom *et al*., 2007). A wide variety of aerobic microorganisms have MTBE degradation capabilities, including fungi and both gram-negative and gram-positive bacteria. Aerobic MTBE degradation has been observed with MTBE used as a primary carbon source or co-metabolically in the presence of another carbon source, such as butane or ethanol. Co-metabolism allows more rapid growth and, thus, more rapid utilization of MTBE, however studies have shown that MTBE degradation ability is lost when the primary substrate is depleted

(Garnier *et al*., 1999).

The primary degradation step in aerobic MTBE degradation is usually oxidation to *tert*-butyl alcohol (TBA) and formic acid, initiated by one of several oxygenases including methane monooxygenase (Liu *et al*., 2001), toluene monooxygenases (Vainberg *et al*., 2006), cytochrome P-450 monooxygenases (Steffan *et al*., 1997), propane monooxygenase (Steffan *et al*., 1997; Smith *et al*., 2003), as well as toluene dioxygenase, ammonium monooxygenase, and propylene monooxygenase (Hyman and O'Reilly, 1999). In some cases, this is the only step observed. Other times, mineralization to carbon dioxide occurs, depending on the organisms involved and the growth conditions.

Studies of *in situ* treatment of groundwater have demonstrated aerobic MTBE biodegradation with native organisms (Salanitro *et al*., 2000), addition of laboratory cultured organisms (Salanitro *et al*., 2000; Spinnler *et al*., 2001; Landmeyer *et al*., 2001) and with the addition of air sparging/soil vapor extraction technologies (Wilson, 2003). There have also been a number of technologies developed for remediation of contaminated groundwater through aerobic MTBE degradation. Aerobic MTBE degrading organisms have also been used in bioreactors, and other biological water treatment systems, for aboveground treatment of contaminated water (Fortin and Deshusses, 1999; Stocking *et al*., 2000; Liu *et al*., 2006; Zien *et al*., 2004, 2006).

F. Studies of anaerobic MTBE biodegradation

Aerobic and anaerobic MTBE degradation were each first reported in 1994, however there is currently much less known about the role of anaerobic microbial

communities in the biodegradation of MTBE. No organisms have been identified from any anaerobic MTBE-degrading consortium and no biodegradation mechanism is known. Anaerobic biodegradation of MTBE is an important process because MTBE contamination often occurs concomitantly with contamination with other fuel components. Rapid degradation of these more easily biodegradable compounds is associated with rapid depletion of oxygen, leaving MTBE in an anoxic environment. Fortunately, anaerobic MTBE biodegradation does occur. The first report of anaerobic MTBE degradation was in 1994, in only one of triplicate enrichment cultures, under methanogenic conditions (Mormile *et al*., 1994). Subsequent studies found anaerobic MTBE biodegradation to also occur under nitrate-reducing (Bradley *et al*., 2001a; Fischer *et al*., 2005), manganese(IV)-reducing (Bradley *et al*., 2002), iron (III)-reducing (Finneran and Lovley, 2001; Bradley *et al*., 2001b; Pruden *et al*., 2005), and sulfate-reducing conditions (Somsamak *et al*., 2001, 2006; Bradley *et al*., 2001a; Fischer *et al*., 2005). Most studies attempting to detect anaerobic MTBE degradation found that degradation was frequently only observed in a small percentage of cultures, whether they were replicates using the same inoculum or testing different inocula and different conditions. This demonstrates the recalcitrance of MTBE, and also that anaerobic MTBE biodegradation appears to be a rare process.

As uncommon as it is, MTBE biodegradation has been detected *in situ* and observed in microcosms of sediments, groundwater and bioreactor sludge from 8 different U.S. states and one location in Germany (Table 1.2.). Initial MTBE concentrations used in anaerobic degradation studies ranged from 1.3 to 100 mg/L. Degradation rates for initial MTBE depletion in anaerobic enrichment cultures are slow,

with over 240 days as the minimum time reported for 100% removal of 100 mg/L starting concentration of MTBE and many studies never observing 100% depletion. Although not observed in every study, total transformation of MTBE has been detected under all electron accepting conditions tested except for Mn(IV)-reducing. Most studies do not show complete mineralization of MTBE. Instead, the intermediate product, *tert*-butyl alcohol (TBA), accumulates and is not further degraded. TBA also accumulates during aerobic MTBE degradation under some conditions suggesting that O-demethylation of MTBE is the first step in both processes and that degradation of TBA is often a rate-limiting step for complete degradation of MTBE (see reviews by Deeb *et al*., 2000; Stocking *et al*., 2000; Fayolle *et al*., 2001; Fiorenza and Rifai, 2003). Studies of the health effects caused by TBA suggest potential carcinogenicity similar to that seen in studies of MTBE (Cirvello *et al*., 1995; US EPA, 1997; Sgambato *et al*., 2009), therefore, TBA is not a desireable biotransformation endpoint. Even in the absence of anaerobic TBA biodgradation, however, anaerobic MTBE biodegradation occurs in the environment and it is therefore important to gain a better understanding of this process.

In the study by Somsamak *et al*. (2001) anaerobic enrichment cultures showed loss of MTBE under methanogenic and sulfidogenic conditions and the stoichiometry showed that utilization of the methyl group was ultimately coupled to either methanogenesis or sulfidogenesis, respectively. However, further experiments conducted with specific inhibitors (molybdate and bromoethanesulfonic acid) suggested that the O-demethylation of MTBE to TBA is not catalyzed by either sulfate-reducers or methanogens (Somsamak *et al*., 2005). Addition of the inhibitors did induce a prolonged lag period prior to the initiation of MTBE loss in the cultures, indicating that the

sulfidogenic and methanogenic organisms are involved in the MTBE degradation process, most likely using the products of MTBE degradation. Such community interactions are common and reliance on this cross-feeding may be one of the reasons why cultural isolation of an anaerobic MTBE degrading organism has proven difficult. The initial ether bond breakage in the degradation of MTBE to TBA is an O-demethylation, suggesting the possibility that this step is mediated by acetogenic bacteria. Acetogens are known to be capable of methylotrophic growth by O-demethylation of aromatic compounds (Bache and Pfennig, 1981; Frazer and Young, 1985; Mechichi *et al*., 1999; Taylor, 1983; Dore and Bryant, 1990; Frazer, 1994). This indicates that they could also be able to subsist on the O-methyl substituent of MTBE, and thus possibly mediate the initial ether cleavage and utilization of the methyl group. However, two pure cultures of acetogens, *Acetobacterium woodii* and *Eubacterium limosum,* which are specifically known for the ability to metabolize methyl ethers, have been tested and found to not degrade MTBE (Mormile *et al*., 1994). It is possible that these organisms have the capacity to degrade MTBE, but require the presence of other microbes.

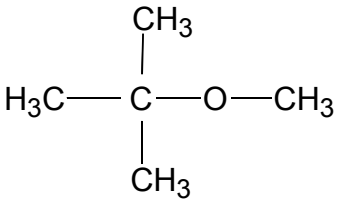
II. Purpose of this study

In this study we analyzed MTBE degrading laboratory cultures using an array of microbiological, molecular and geochemical approaches. The objective was to derive critical information about MTBE degradation mechanisms and the responsible organisms that will influence future endeavors to stimulate and monitor *in situ* MTBE degradation under different environmental conditions. Much of this work to further examine the anaerobic MTBE degradation process and identify responsible organisms used enriched

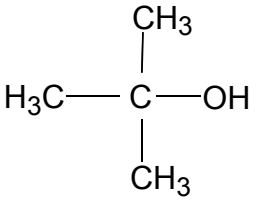
anaerobic MTBE-degrading cultures that had been established years earlier. MTBE contaminated groundwater is an ongoing problem in industrial nations around the world. Information about microbial mediated MTBE transformation processes is critical for continued development of assisted biodegradation processes and for monitoring MTBE degradation in the environment.

The specific objectives of this study were:

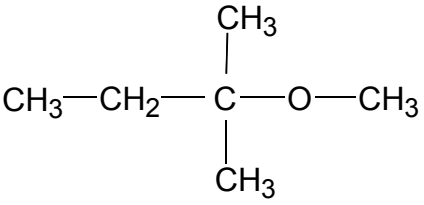
1. To examine the effects of cultural amendments on the anaerobic MTBE degradation process of anaerobic enrichment cultures;
2. To use molecular community analysis techniques to collect phylogenetic information about the community composition of anaerobic MTBE degrading enrichment cultures;
3. To investigate the carbon flow within MTBE degrading enrichment cultures using stable isotope probing techniques;
4. To study the carbon and hydrogen stable isotope fractionation during MTBE degradation in anaerobic enrichment cultures;
5. To study the carbon and hydrogen stable isotope fractionation during aerobic MTBE degradation by the cold-active bacterium, *Variovorax paradoxus.*



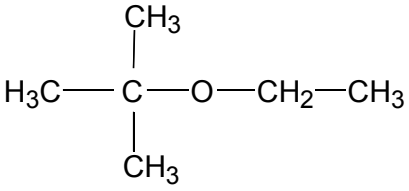
methyl *tert*-butyl ether
(MTBE)



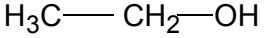
tert-butyl alcohol
(TBA)



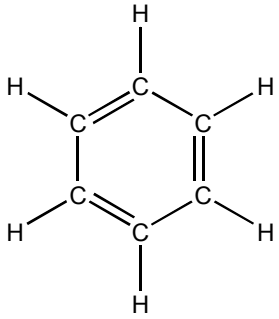
tert-amyl methyl ether
(TAME)



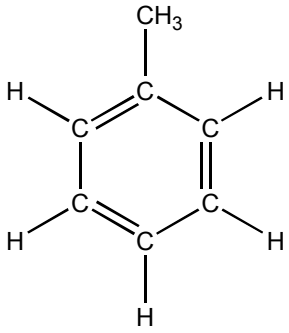
ethyl *tert*-butyl ether
(ETBE)



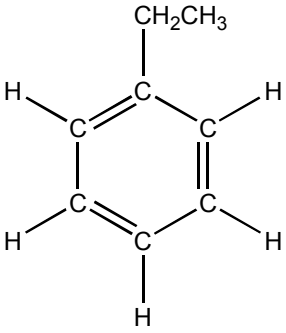
ethanol



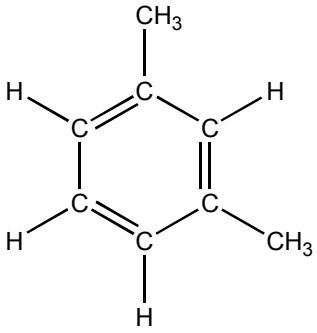
benzene



toluene



ethylbenzene



m-xylene

FIGURE 1.1. Structures of MTBE and other fuel oxygenates and components.

TABLE 1.1. Properties of fuel components.

Compound	Water solubility [1] (mg/L)	Vapor pressure [2] (mm Hg)	Henry's Law Constant [3]	Log K_{oc} [4]
MTBE	50,000	251	0.055	1.1
ETBE	26000	152	0.11	1.6
TAME	20000	68	0.052	1.7
TBA	Infinite	41	0.00049	1.6
Ethanol	Infinite	53	0.00024	0.71
Benzene	1780	86	0.22	1.9
Toluene	535	28	0.24	1.9
Ethylbenzene	161	10	0.35	2.7
m-xylene	146	8.3	0.31	2.3
o-xylene	175	6.6	0.21	1.8
p-xylene	156	8.7	0.31	2.4

Table adapted from (Moyer, 2003)

[1] indicates tendency to dissolve in water

[2] indicates tendency to volatilize from the non-aqueous phase

[3] indicates tendency to volatilize from aqueous phase

[4] indicates tendency to adhere to soil

TABLE 1.2. Microcosm studies demonstrating anaerobic MTBE biodegradation.

Inoculum source	Anaerobic condition	MTBE concentration, incubation time, and extent of degradation	Number of microcosms showing degradation vs. not showing degradation	Reference
Oligotrophic soil, Virginia	Methanogenic	100 mg l^{-1}, 270 days, 80-100%	Three sites tested under three conditions. Degradation only at one site under one condition	Yeh and Novak, 1994
River sediment, Ohio	Methanogenic	48 mg l^{-1}, 152 days, 46%	Degradation only observed in one of triplicate identically prepared microcosms	Mormile *et al*., 1994
Aquifer material, South Carolina	Fe(III) reducing	U-^{14}C-MTBE, 73,000-666,000 dpm, 7 months, 3% production of radiolabeled CO_2 .	Several conditions tested, MTBE degradation only seen under one	Landmeyer e*t al*., 1998
Aquifer material, North Carolina	Methanogenic	3.1-5.7 mg l^{-1}, 490 -590 days, 99%	MTBE degradation in both alkylbenzene-supplemented and unsupplemented culture conditions.	Wilson *et al*., 2000
Surface water sediment, South Carolina, Florida, New Jersey	Methanogenic, Sulfate-reducing, Nitrate-reducing, Fe(III)-reducing, Mn(IV)-reducing	U-^{14}C-MTBE, 1.3-1.6 mg l^{-1}, 166 days, 10-80%	80% MTBE mineralization in Florida sediments under sulfate-reducing conditions. Only 10-20% mineralization at other sites and other anaerobic conditions	Bradley *et al*., 2001a
Streambed sediment, South Carolina	Nitrate-reducing	U-^{14}C-MTBE 1.5-1.8 mg l^{-1}, 77 days, 25%	Significant MTBE mineralization seen under nitrate-reducing conditions, but not under methanogenic or sulfate-reducing	Bradley *et al*., 2001b

TABLE 1.2. (continued)

Inoculum source	Anaerobic condition	MTBE concentration, incubation time, and extent of degradation	Number of microcosms showing degradation vs. not showing degradation	Reference
MTBE-contaminated aquifer sediment, South Carolina	Fe(III) Reducing	50 mg l^{-1}, 275 days, 100%	MTBE degradation seen in 1 out of 5 conditions tested. Only one of 3 Fe(III)-reducing replicates showed degradation	Finneran and Lovely, 2001
Estuarine sediment, New Jersey, New York	Sulfate reducing	100 mg l^{-1}, 1160 days, 100%	MTBE degradation only under sulfate reducing conditions (out of 4 conditions tested) and only in some replicates. No MTBE loss observed in methanogenic, nitrate-reducing, or Fe(III)-reducing cultures	Somsamak *et al.*, 2001
Aquifer material, New Jersey	Unidentified	~9 mg l^{-1}, 199 days, 10-99%	MTBE degradation seen in 5 out of 12 replicates	Kolhatkar *et al.*, 2002
Estuarine sediment, New Jersey, New York, California	Sulfate reducing, Methanogenic	100 mg l^{-1}, 246-1160, 100%	3 out of 9 sites tested showed degradation in 1 out of 3 replicates of each..	Somsamak, 2005 Somsamak *et al.*, 2005, 2006
Bioreactor sludge, Texas	Fe(III) reducing	5 mg l^{-1}, 380 days, 100%	Similar results for all 72 active microcosms	Pruden *et al.*, 2005
Groundwater samples, contaminated wells, Leuna, Germany	Sulfate reducing, Nitrate reducing	~50 mg l^{-1}, 180 days, 60%	Out of 20 microcosms, only 1 sulfate-reducing and 3 nitrate-reducing cultures showed MTBE degradation	Fischer *et al.*, 2005

Chapter 2

Effects of co-substrates and inhibitors on anaerobic methyl *tert*-butyl ether (MTBE) degradation

Published in Applied Microbiology and Biotechnology (2008) 80: 1113-1120.Effects of co-substrates and inhibitors on the anaerobic O-demethylation of methyl tert-butyl ether (MTBE), Youngster, L. K. G., P. Somsamak, and M. M. Häggblom.

I. Abstract

Methyl *tert*-butyl ether (MTBE) contamination is widespread in aquifers near urban areas around the world. Since this synthetic fuel oxygenate is resistant to most physical methods of treating fuel-contaminated water, biodegradation may be a useful method of remediation. Currently, information on anaerobic MTBE degradation is scarce. Depletion has been observed in soil and sediment microcosms from a variety of locations and under several redox conditions, but the responsible organisms are unknown. We are studying anaerobic consortia, enriched from contaminated sediments for MTBE-utilizing microorganisms for over a decade. MTBE degradation occurred in the presence of other fuel components and was not affected by toluene, benzene, ethanol, methanol, or gasoline. Many aryl O-methyl ethers, such as syringic acid, that are O-demethylated by acetogenic bacteria, were also O-demethylated by the MTBE-utilizing enrichment cultures. The addition of these compounds as co-substrates increased the rate of MTBE-degradation, offering a potentially useful method of stimulating the MTBE-degradation rate *in situ*. Propyl iodide caused light-reversible inhibition of MTBE-degradation, suggesting that the MTBE degradation process is corrinoid-dependent. The anaerobic MTBE-degradation process was not directly coupled to methanogensis or sulfidogenesis and was inhibited by the bactericidal antibiotic, rifampicin. These results suggest that MTBE-degradation is mediated by acetogenic bacteria.

II. Introduction

MTBE is a synthetic volatile organic compound, which when added to gasoline, increases octane and reduces hazardous combustion emissions. The U.S. Clean Air Act amendments of 1990 mandated the use of such fuel oxygenates in polluted urban areas to improve air quality (Franklin *et al*., 2000). Due to its low production cost and ease of blending with gasoline (Shelly and Fouhy, 1994) MTBE was the most frequently used fuel oxygenate between 1990 and 2002. In 1995, 30% of the fuel in the United States was formulated to include up to 15% MTBE by volume and MTBE was produced at the second highest volume of any synthetic organic chemical (US EPA, 1999, 2000). Production in the U.S. peaked in 1999 at over 9200 million kg/year (EIA/DEO, Häggblom *et al*., 2007). Unfortunately, as production increased, MTBE emerged as a frequent water contaminant (Squillace *et al*., 1996, 1999; Pankow *et al*., 1997; Dernbach, 2000; Johnson *et al*., 2000; Achten *et al*., 2002a, 2002b; Ayotte *et al*., 2005; Reuter *et al*., 1998; Toran *et al*., 2003; Heald *et al*., 2005). As MTBE contamination gained notoriety as a persistent environmental problem, many US states banned or restricted MTBE use in fuel (US EPA, 2006). Though production and use of MTBE have decreased considerably, MTBE contamination is a persistent and widespread problem that requires remediation.

The US EPA considers MTBE to be a possible human carcinogen based on limited animal evidence (US EPA, 1993). There is now evidence that tumor development in rats can be clearly linked to MTBE exposure and the carcinogenic effect is likely relevant to humans (Caldwell *et al.*, 2008). MTBE is also a skin and respiratory irritant and causes reproductive mutations in zebrafish at concentrations often reported in

contaminated environments (Werner *et al*., 2001; Moreels *et al*., 2006b). Currently the US EPA recommends that drinking water contain no more than 25 ppb MTBE based on aesthetic concerns (US EPA, 1997). MTBE can be detected by taste and odor at as low a concentration as 1 ppb. Many states have adopted lower thresholds of 13-14 ppb because any greater concentration renders water unpalatable (Ayotte *et al*., 2005).

Extensive groundwater contamination with MTBE is problematic due to taste, odor, and health concerns. MTBE has been detected in over 1850 aquifers in 29 U.S. states and several municipal water supplies have been closed due to contamination with MTBE (Environmental Working Group, 2005; US EPA, 2006). MTBE enters water through spills and leaks during production, transportation, storage, use, and disposal, or indirectly through volatilization and precipitation and storm water runoff (Squillance *et al*., 1996; Reuter *et al*., 1998; Brown *et al*., 2000). It has been estimated that between $4 and 85 billion will be required to clean up MTBE-contaminated water supplies for public water systems in the United States (AWWA, 2005).

Contamination of groundwater with MTBE presents a challenge to remediation efforts, as its physical characteristics make it more persistent and mobile in groundwater than other common components of gasoline. High water solubility and a low Henry's Law constant make MTBE more prone to dissolution in water and rapid migration throughout the water body once dissolved (Squillance *et al*., 1997). It is also less likely to be hindered by volatilization or adherence to soil or carbon-based filters (Stocking *et al*., 2000). These properties mean that innovative methods for MTBE removal are required since MTBE is not efficiently removed by common methods of treating fuel-contaminated water (US EPA, 2004).

The tertiary carbon structure and stable ether bond make MTBE resistant to microbial transformation. Initially thought to be entirely recalcitrant to biodegradation, there have now been many reports of MTBE-biodegradation in both aerobic (Salanitro, 1994; Deeb *et al*., 2000; Stocking *et al*., 2000; Fayolle *et al*., 2001) and anaerobic environments (Suflita and Mormile, 1993; Mormile *et al*., 1994; Wilson *et al*., 2000; Somsamak *et al*., 2001, 2005, 2006; Bradley *et al*., 2001a, 2002; Finneran and Lovely, 2001; Fischer *et al*., 2005; Pruden *et al*., 2005). Aerobic MTBE-utilizing organisms have been isolated and studied, but much less is known about anaerobic MTBE-biodegradation. Anaerobic MTBE degradation occurs under a variety of redox conditions (Somsamak *et al*., 2001, 2005, 2006; Bradley *et al*., 2001a; Pruden *et al*., 2005; Bradley *et al*., 2002), but the responsible organisms and mechanism are unknown. O-demethylation to *tert*-butyl alcohol (TBA) is the initial degradation step in all reports of anaerobic microbial transformation.

For anaerobic biodegradation to be a reliable method of natural attenuation of contaminated aquifers, we need further information about the process and how it can be affected by environmental conditions. Through strategic addition of co-substrates and inhibitors to the MTBE-utilizing enrichment cultures, we have uncovered information about anaerobic MTBE degradation. Amendments were selected to replicate likely combinations of contaminants present in polluted environments, increase the rate of MTBE-degradation, or produce degradation effects that suggest characteristics of the responsible MTBE-degrading organisms and mechanisms.

III. Materials & Methods

A. Enrichment cultures and growth conditions

Sediment collected from different sites were previously used to establish anaerobic enrichment cultures as described (Somsamak *et al.*, 2001, 2005). Cultures originating from the Arthur Kill Inlet (AK) between New Jersey and New York, or the New York Harbor (NY) were maintained using strict anaerobic technique under methanogenic or sulfidogenic conditions and were repeatedly transferred into fresh medium and enriched with MTBE as the sole carbon source. Select enrichment cultures, representing 10^{-3} to 10^{-5} transfers of the original enrichments, were chosen for different experiments to study the effect of co-substrates and inhibitors on MTBE degradation. All experiments were conducted in 10 to 50 mL glass serum vials capped with Teflon-coated stoppers and aluminum seals. Cultures were incubated at 28°C and the concentration of MTBE was monitored regularly as described below. All experiments were set up in triplicate and included abiotic controls that consisted of cell-free media, spiked with MTBE and maintained under the same conditions as live cultures.

B. Effects of gasoline co-contaminants

The effect of likely groundwater co-contaminants on MTBE-degradation was tested in sulfidogenic AK cultures spiked to a concentration of 300 µM MTBE (Aldrich, Milwaukee, WI). Subsets were additionally spiked with either 20 µM benzene (EM Science, Toronto, Canada), or 80 µM toluene (JT Baker, Phillipsburg, NJ), methanol (Fisher, Fair Lawn, NJ), or ethanol (Aldrich, Milwaukee, WI). When complete use of MTBE occurred in each individual culture, biosolids were allowed to settle by gravity,

after which media and accumulated TBA were carefully removed by syringe, and fresh media was added. The cultures were then re-spiked with the same combination and same initial concentrations of MTBE and co-substrates.

In a separate experiment, 50 mL cultures were spiked with MTBE to a concentration of 200 μM and a subset was additionally spiked with 6.8 μL regular unleaded MTBE-free gasoline (Delta fuel station, Piscataway, NJ) to give a ratio of 15% MTBE:85% gasoline by volume relative to the 200 μM MTBE added to a 50 mL culture.

C. Effects of methoxylated aromatic compounds on MTBE degradation

A methanogenic AK enrichment was used to determine the effects of different concentrations of syringate on MTBE-degradation. Cultures were spiked with 85 μM MTBE and syringate (4-hydroxy-3,5-dimethoxybenzoic acid) (Sigma, St Louis, MO) to a concentration of either 0 μM, 50 μM, 100 μM, 500 μM, 750 μM, or 1000 μM. Degradation of MTBE and syringate were monitored over time.

A sulfidogenic AK enrichment culture was used to determine the effects of different methoxylated aromatic compounds on the rate of anaerobic MTBE-degradation. Cultures were spiked to a concentration of 400 μM MTBE. Subsets were additionally spiked to a concentration of 50 μM of either syringate, guaiacol (2-methoxyphenol) (Sigma, St Louis, MO), vanillate (4-hydroxy-3-methoxybenzoic acid) (Alfa Aesar, Ward Hill, MA), anisole (methoxybenzene) (TCI, Portland, OR), veratrol (1,2-dimethoxybenzene) (Acros Organics, Morris Plains, NJ), 3,4,5-trimethoxybenzoate (Acros Organics, Morris Plains, NJ), or ferulate (3-(4-hydroxy-3-methylphenyl)-2-propenoic acid) (Indofine, Hillsborough, NJ). When full MTBE loss had occurred in

cultures amended with syringic acid, guaiacol or vanillate, media was replaced and the cultures were re-spiked with the same combination and same initial concentrations of MTBE and co-substrate. 3,4,5-Trihydroxybenzoate, 3,4-dihydroxybenzoate, and catechol (MP Biomedicals, Solon, OH) were used as standards for identifying metabolites.

D. Effects of inhibitors

To investigate the effects of propyl iodide on anaerobic MTBE-degradation, we used a methanogenic enrichment culture from NY sediment. All cultures were spiked with MTBE to a final concentration of 100 μM. Propyl iodide (TCI, Portland, OR) was added to a half of the enrichment culture vials to a concentration of 20 μM. One set of cultures was incubated in constant light and another set was incubated in the dark.

To examine the effects of rifampicin on anaerobic MTBE-degradation, both methanogenic and sulfidogenic NY enrichment culture were used. All cultures were spiked with MTBE to a final concentration of 150 μM with or without 12 μM rifampicin (Sigma, St. Louis, MO).

E. Analytical methods

Concentrations of MTBE, *tert* butyl alcohol, methanol, ethanol, benzene, toluene, and gasoline were determined, as described in Somsamak *et al.* (2001), through direct aqueous injection of a sample volume of 1μl using a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector (GC-FID). Compounds were separated on a DB1 capillary column (0.53mm x 30m, J&W Scientific, Folsom, CA).

Concentrations of methoxylated aromatic compounds were measured on an Agilent 1100 series high-performance liquid chromatograph equipped with a reversed-phase Sphereclone column (5µ ODS(2), 250mm x 4.60mm; Phenomenex, Torrance, CA). Compounds were eluted with a linear MeOH-H_2O gradient (0.1% acetic acid) in which the MeOH concentration was increased from 35 to 65% at a column temperature of 30°C and a flow rate of 1ml/min.

IV. Results

A. Effects of gasoline co-contaminants on anerobic MTBE degradation

MTBE contamination typically occurs in combination with other fuel components. We therefore determined the effects of representative gasoline compounds on anaerobic MTBE degradation. Sulfidogenic AK enrichment cultures were spiked with MTBE and either methanol, benzene, toluene, ethanol, or gasoline at concentration ratios of MTBE to fuel/fuel component selected to mimic those that might be found in a fuel spill. MTBE was degraded in all cultures and none of these compounds had a substantial effect on the degradation rate or lag period (Table 2.1.), except for a slightly increased rate of degradation upon repeated addition of methanol.

B. Effects of methoxylated aromatic compounds on MTBE degradation

The initial step in anaerobic MTBE degradation is an O-demethylation of MTBE to form TBA. Since several acetogenic bacteria use the O-methyl group from methoxylated aromatic compounds (Bache and Pfennig, 1981; Frazer and Young, 1985; Mechichi *et al*., 1999; Taylor, 1983; Dore and Bryant, 1990; Frazer, 1994), we therefore

determined the effect of these methoxylated aromatics as potential co-substrates for MTBE degrading bacteria.

When MTBE was added along with several different concentrations of syringate to a methanogenic AK culture, we found that low concentrations of syringate (50 μM to 500 μM) increased the MTBE degradation rate, while concentrations of 750 μM and greater decreased the MTBE degradation rate. The MTBE degradation rate was highest with 50 μM of syringate (Figure 2.1.). Addition of 50 μM of 3,4,5-trimethoxybenzoate or veratrol also increased the rate of MTBE depletion. Addition of ferulate and anisole did not have a significant impact on the degradation rate, while guaiacol and vanillate increased the rate of MTBE loss (Table 2.2.). O-demethylation and decarboxylation of the methoxy aromatic compounds and formation of metabolites occurred in the anaerobic MTBE-utilizing enrichment cultures during the lag period before MTBE degradation commenced (Figure 2.2.). After repeated spikings with both MTBE and 50 μM co-substrate, addition of syringate, vanillate, or guaiacol increased the rate of MTBE degradation compared to the degradation rate of cultures that were repeatedly spiked with MTBE alone (Figure 2.3.).

C. Inhibitor studies

Many anaerobic O-demethylation pathways require a corrinoid-containing protein to act as the methyl acceptor (Stupperich and Kräutler, 1988; Kaufman *et al*., 1997; Naidu and Ragsdale, 2001). Propyl iodide inhibits corrinoid-dependent O-demethylation by binding the corrinoid in a light-reversible manner and preventing it from participating in the reaction (Ghambeer *et al*., 1971; Choi *et al.*, 1994). In the methanogenic NY

enrichment cultures, MTBE degradation was inhibited by propyl iodide in the dark and inhibition was reversed by incubation under light (Figure 2.4.), suggesting the involvement of a corrinoid mediated reaction.

We have previously reported that addition of specific inhibitors of methanogenesis and sulfidogenesis, bromoethanesulfonic acid and molybdate, do not inhibit MTBE degradation in methanogenic and sulfidogenic enrichment cultures, respectively (Somsamak *et al*., 2005). To further illustrate that the MTBE biodegradation process is not mediated by methanogenic archaea, we use a bacterial inhibitor, rifampicin. This antibiotic inhibits bacterial RNA polymerase, but is ineffective on archaea and therefore inhibits acetogens and other bacteria, but not archaea (Bräuer *et al*. 2004). MTBE degradation was completely inhibited in the presence of rifampicin, while degradation of MTBE occurred normally in rifampicin-free controls (Figure 2.5.).

V. Discussion

Here we present new information about anaerobic MTBE degradation in sediment enrichment cultures and how this process can be enhanced. The prevalence of anoxic conditions in gasoline polluted groundwater means that anaerobic MTBE degradation will likely be a necessary process of remediation. MTBE degradation is a rare process and the responsible organisms are sparsely distributed, even in contaminated sediments. This is demonstrated by the many studies that have tested for anaerobic MTBE degradation and either yielded negative results or observed degradation in only a few of many established cultures (Suflita and Mormile, 1993; Borden *et al*., 1997; Chen *et al*., 2005). Our findings suggest that anaerobic MTBE degradation in the enrichment cultures

is likely mediated by acetogenic bacteria. *Eubacterium limosum* and *Acetobacterium woodii*, two O-demethylating acetogens, were previously tested and found unable to degrade MTBE (Mormile *et al*., 1994). Several attempts to isolate an MTBE degrading species from the enrichment cultures by cultural means have been unsuccessful. It is possible that MTBE degradation requires the interactions of a consortium. Other microbial degradation processes are dependent on community interactions (Jimènez *et al.*, 1991; Lappin *et al*., 1985; Rozgaj *et al*., 1992) and there is a precedent for symbiotic relationships between methanogens and acetogens leading to improved ability to conduct degradation reactions that would otherwise not be energetically efficient (Conrad *et al.*, 1985). Previous studies have indicated that while MTBE degradation is not directly coupled to methanogenesis or sulfidogenesis, inhibitors of these processes substantially slow the rate of MTBE degradation. This suggests that while neither methanogens nor sulfidogens are directly capable of demethylating MTBE, these facilitate MTBE degradation, perhaps by utilizing the products of the acetogenic metabolism. Therefore, it is essential that we study the factors that influence the degradation process as it occurs in the mixed community of our enrichment cultures.

The MTBE degradation rate in the sediment enrichment cultures was unaffected by co-spiking with fuel components and gasoline. Since contamination with MTBE and gasoline are usually concurrent, anaerobic MTBE degradation would not be a practical remediation tool if it were significantly hindered by the presence of gasoline. The slight increase in the MTBE-degradation rate by methanol suggests that the MTBE demethylating organisms are also able to use methanol as a carbon source. The data is in accordance with previous findings that growth rates of *Eubacterium limosum*, an

acetogen with O-demethylating capabilities, were similar on methanol, vanillate, or syringate (Cocaign *et al.*, 1991).

Lack of degradation activity in cultures with rifampicin supports previous data that the degradation reaction is not mediated by methanogenic archaea (Somsamak *et al.*, 2005). While MTBE degradation occurs when methanogenesis and sulfidogenesis are inhibited, degradation is completely halted by the bacterial inhibitor, rifampicin. This indicates that the MTBE degradation process requires bacteria, although the data does not preclude the involvement of other microorganisms. Results of the propyl iodide experiment provide information about the pathway of anaerobic MTBE degradation. The light-reversible inhibition in the presence of propyl iodide suggests that the O-demethylation of MTBE to TBA may be a corrinoid dependent reaction. Acetogenic and many methanogenic demethylation reactions are corrinoid dependent. Since a methanogenic pathway is ruled out by the rifampicin study, as well as by data from a previous paper (Somsamak *et al*., 2005), the evidence implies that the pathway is corrinoid dependent and likely mediated by acetogenic bacteria. We are currently investigating how the MTBE degrading community diversity changes with continued enrichment and exposure to the co-substrates.

It is also propitious that we find an increased MTBE degradation rate with methoxylated aromatic acids as co-substrates (Figures 2.1.-2.3.). Syringic acid, ferulate, guaiacol, anisole, vanillate, veratrol, and 3,4,5-trimethoxybenzoate are naturally occurring lignoaromatic compounds that could conceivably be added at low concentrations to contaminated environments to stimulate MTBE degradation activity. Enhancement of MTBE degradation by addition of O-methoxylated aromatic compounds

also supports the hypothesis that the anaerobic MTBE-degradation reaction may be mediated by acetogenic bacteria. Acetogens are known to degrade the methoxylated aromatics (Bache and Pfennig, 1981; Frazer and Young, 1985; Mechichi *et al*., 1999; Taylor, 1983; Dore and Bryant, 1990; Frazer, 1994) and our cultures were also able to degrade many of these compounds. Syringate and 3,4,5-trimethoxybenzoate immediately increased the rate of anaerobic MTBE degradation and several of the other methoxylated aromatic compounds increased the degradation rate after repeated exposure. The enrichment cultures use the methyl group from the aromatic compounds before they begin to appreciably O-demethylate MTBE (Figure 2.2.). Enzymes catalyzing the O-demethylation reaction may have a greater affinity for the naturally occurring compounds than for MTBE, a synthetic chemical. If the organisms in the culture have evolved to subsist off of O-methyl substituants due to the selective pressures of the enrichment conditions, lignoaromatic co-substrates may increase the rate of MTBE degradation because O-demethylation of MTBE has a higher activation energy than that O-demethylation of an aromatic compound because the aromatic ring creates increased stability in the demethylated aromatic product. In either case, the addition of a low concentration of the naturally occurring methoxylated compounds may be a useful way of enhancing and possibly stimulating anaerobic MTBE degradation *in situ*. Future studies will investigate the use of the O-methylated aromatic compounds presented here to stimulate or enhance MTBE degradation *in situ* in polluted environments.

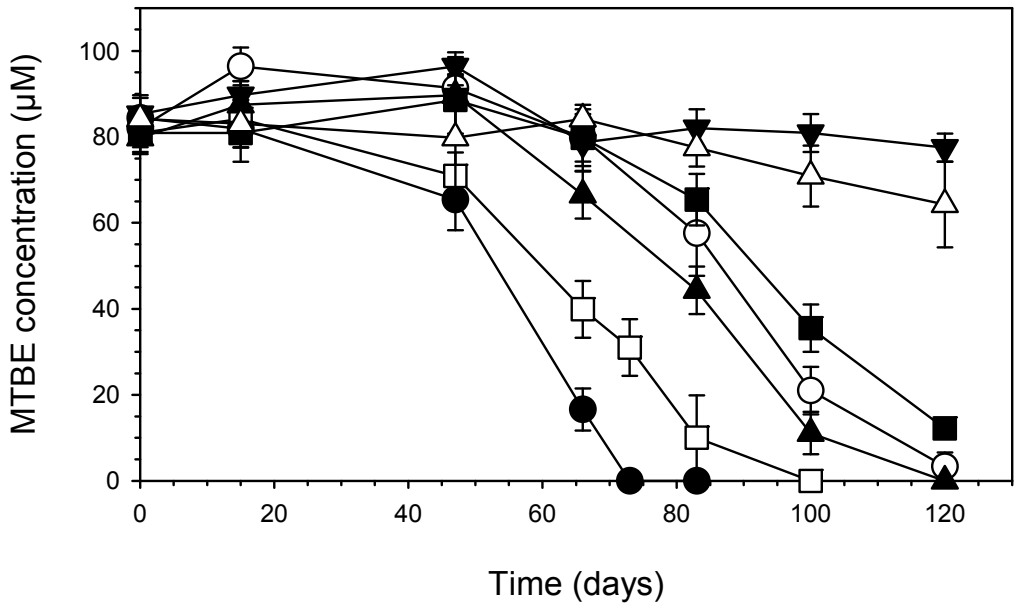


FIGURE 2.1. Effect of different concentrations of syringate on MTBE degradation. MTBE alone (○) or MTBE + 50 µM syringate (●), 100 µM syringate (□), 500 µM syringate (▲), 750 µM syringate (■) or 1000 µM syringate (Δ) and an abiotic control (▼) Error bars represent the mean and standard deviation of triplicate cultures.

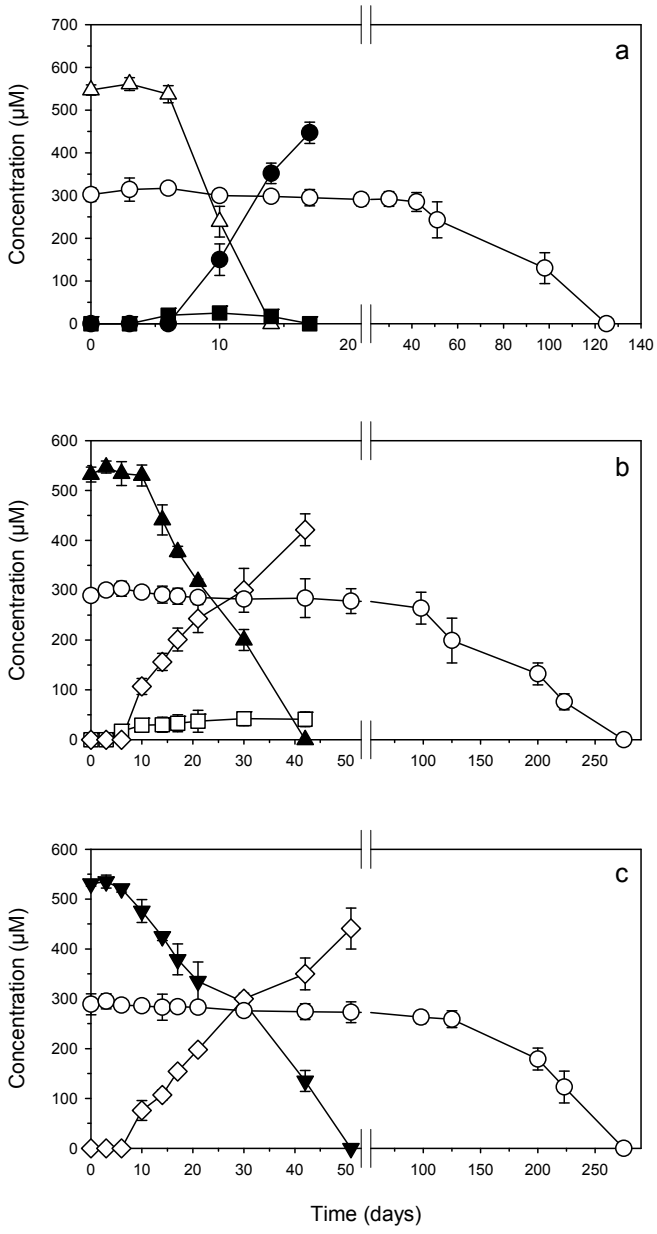


FIGURE 2.2. Anaerobic degradation of MTBE (○) and (a) syringate (Δ), (b) vanillate (▲), or (c) guaiacol (▼) and production of metabolites 3,4,5-trihydroxybenzoic acid (■), 1,2,3-trihydroxybenzene (●), 3,4-dihydroxybenzoic acid (□) and 1,2-dihydroxybenzene (◊). Error bars represent the mean and standard deviation of triplicate cultures.

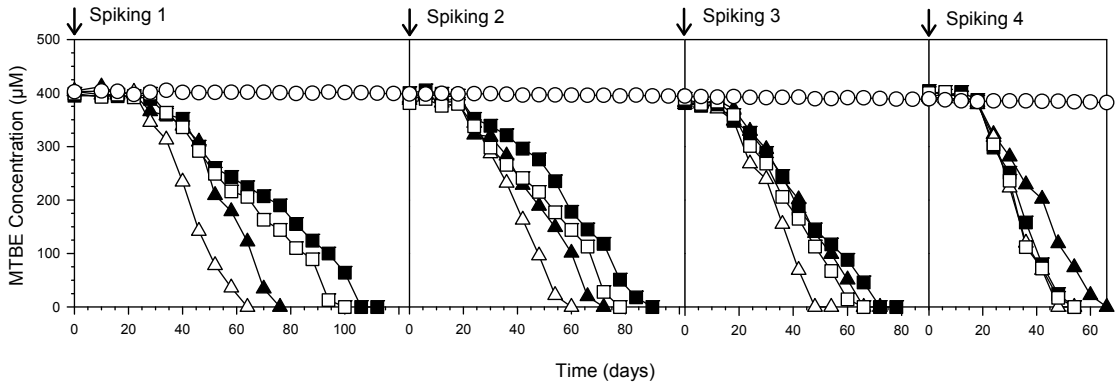


FIGURE 2.3. Effects of repeated spikings with O-methylated co-substrates on MTBE degradation. Concentrations of MTBE were monitored following 4 sequential spikings with either 400 μM MTBE alone or in combination with 50 μM of a co-substrate. MTBE alone (▲), MTBE + syringate (Δ), MTBE + guaiacol (■), MTBE + vanillate (□), and an abiotic control (○). Error bars represent the mean and standard deviation of triplicate cultures.

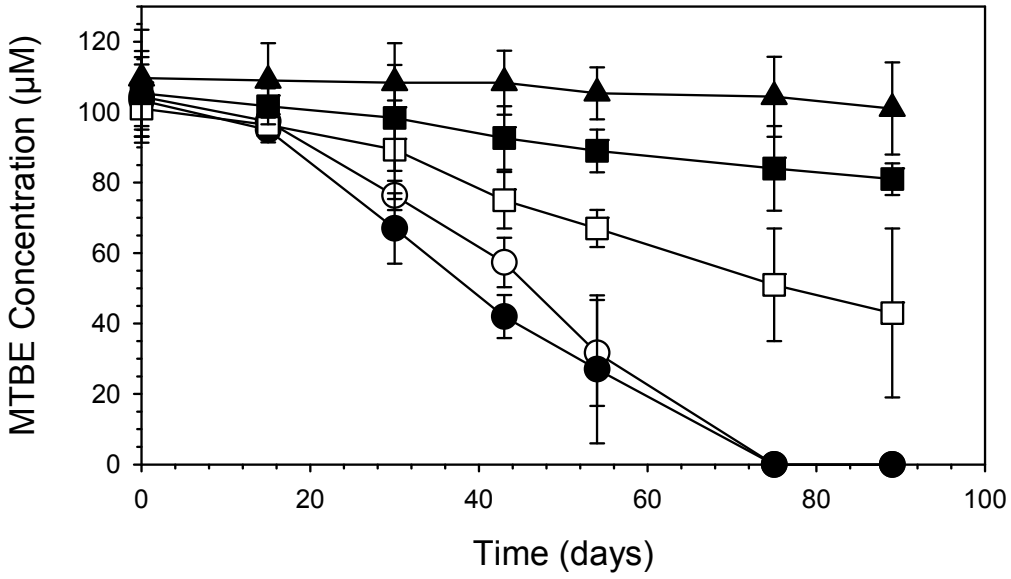


FIGURE 2.4. Light-reversible inhibition of MTBE degradation by propyl iodide. Concentrations of MTBE: exposed to light (○), in darkness (●), + propyl iodide exposed to light (□), + propyl iodide in darkness (■), abiotic control (▲). Error bars represent the mean and standard deviation of triplicate cultures.

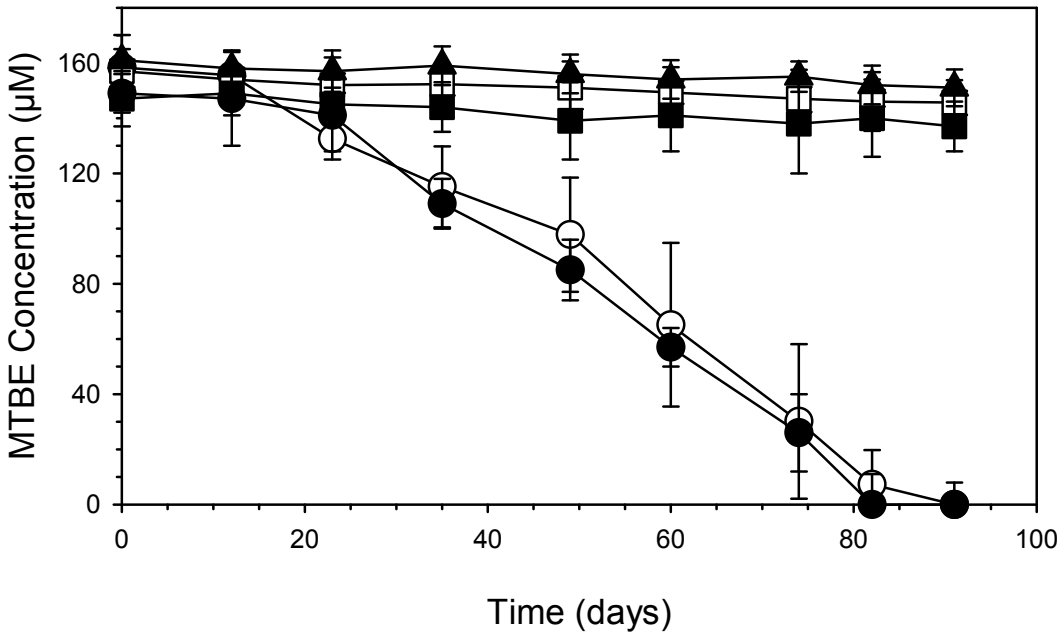


FIGURE 2.5. Inhibition of MTBE degradation by rifampicin. Concentrations of MTBE in methanogenic cultures spiked with MTBE (○) or MTBE + rifampicin (□), or sulfidogenic cultures spiked with MTBE (●) or MTBE + rifampicin (■) and an abiotic control (▲). Error bars represent the mean and standard deviation of triplicate cultures.

TABLE 2.1. Rates of MTBE degradation following either the first or third spiking with either MTBE alone or with a gasoline compound. Data represents the mean and standard deviation of triplicate samples.

Co-Substrate	Spiking	Rate of MTBE loss (μM/L)/Day
None	1	7.0 +/- 0.5
	3	6.6 +/- 0.2
Toluene	1	6.6 +/- 0.8
	3	7.1 +/- 0.5
Methanol	1	6.4 +/- 0.2
	3	8.6 +/- 0.9
Benzene	1	7.4 +/- 0.3
	3	7.8 +/- 0.6
Ethanol	1	8.2 +/- 0.6
	3	7.3 +/- 0.7
Gasoline	1	6.3 +/- 0.7

TABLE 2.2. Rates of MTBE degradation following either the first or fourth spiking with either MTBE alone or with a methoxylated aromatic compound. Data represents the mean and standard deviation of triplicate samples.

Co-Substrate	Spiking	Rate of MTBE loss (μM/L)Day
None	1	7.8 +/- 0.4
	4	8.0 +/- 0.2
Syringate	1	10.4 +/- 0.2
	4	10.7 +/- 0.4
Guaiacol	1	4.6 +/- 0.2
	4	10.7 +/- 0.7
Vanillate	1	5.4 +/- 0.2
	4	10.9 +/- 0.2
Anisole	1	6.8 +/- 0.5
Veratrol	1	9.8 +/- 0.4
Trimethoxybenzoate	1	12.8 +/- 0.5
Ferulate	1	9.0 +/- 0.4

Chapter 3

Community characterization of anaerobic methyl *tert*-butyl ether (MTBE) degrading enrichment cultures

I. Abstract

Use of the synthetic fuel oxygenate methyl *tert*-butyl ether (MTBE) has led to widespread environmental contamination. Anaerobic biodegradation of MTBE is a potential means for remediation of contaminated aquifers and has been observed under different redox conditions, however no responsible microorganisms have yet been identified. We analyzed the microbial communities in anaerobic enriched cultures from different contaminated sediments that have retained MTBE-degrading activity for over a decade. In these cultures MTBE is transformed to *tert*-butyl alcohol and the methyl group used as a carbon and energy source. Terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial 16S rRNA genes showed that the MTBE-utilizing microcosms established from different sediment sources had substantially different community profiles, suggesting that there are likely multiple species capable of MTBE biodegradation. The 16S rRNA genes from one enrichment culture were cloned and sequenced. Phylogenetic analysis showed a diverse population, with phylotypes belonging to the *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Chloroflexi* and *Thermotogae*. Continued enrichment on MTBE further reduced the population to three predominant phylotypes, as evidenced by T-RFLP analysis, which were most closely related to the *Deltaproteobacteria*, *Firmicutes* and *Chloroflexi*. Identification of the microorganisms mediating anaerobic MTBE degradation will provide the foundation for developing tools for site assessment and bioremediation monitoring.

II. Introduction

Methyl *tert*-butyl ether (MTBE) is a volatile colorless liquid, synthesized from methanol and isobutylene. First used in the late 1970s, MTBE was produced at the second highest volume of any synthetic organic chemical by 1995 and production in the United States peaked in 1999 at over 9,200 million kg/year (US EPA, 1999; EIA/DEO, Häggblom *et al*., 2007). Most of this MTBE was mixed with gasoline to increase octane and improve air quality by reducing hazardous combustion emissions. In the U.S., one third of gasoline included up to 15 percent MTBE by volume, as required for fuel formulations in polluted urban areas by the U.S. Clean Air Act amendments of 1990 (Franklin *et al*., 2000; US EPA, 2000). Over many years of heavy use, MTBE has become a significant contaminant in groundwater, requiring remediation due to its persistence in the environment (Squillace *et al*., 1996, 1999; Pankow *et al*., 1997; Dernbach, 2000; Johnson *et al*., 2000; Achten *et al*., 2002a, 2002b; Ayotte *et al*., 2005; Reuter *et al*., 1998; Toran *et al*., 2003; Heald *et al*., 2005). Production and use of MTBE in the U.S. began to decline in 2003 when many U.S. states banned or restricted the use of MTBE in fuel.

In contrast to other fuel components, MTBE is highly water soluble and has a low Henry's Law constant and soil adsorption coefficient (Squillace *et al*., 1997; Stocking *et al*., 2000). These properties cause MTBE to migrate rapidly throughout a water system once dissolved and make common fuel spill remediation techniques less efficient for removing MTBE (US EPA 2000). Over 1850 aquifers in 29 U.S. states have reported MTBE contamination, sometimes resulting in municipal water supply closings (Environmental Working Group, 2005; US EPA, 2006). Estimated costs of MTBE

cleanup from public United States water supplies range from $4 to 85 billion (AWWA, 2005). Current guidelines for MTBE regulations in drinking water reflect that MTBE is a skin and respiratory irritant and has an unpleasant taste and odor which can be detected at concentrations as low as 1 ppb (Werner *et al*., 2001). Recent studies have concluded that MTBE exposure resulted in tumor development in rats (Caldwell *et al.*, 2008), strengthening the US EPA's initial assessments of MTBE as a possible human carcinogen (US EPA, 1993). In addition to human health issues, MTBE poses ecological concerns since it has been found to cause reproductive mutations in zebrafish at concentrations often reported in contaminated environments (Moreels *et al*., 2006b).

The tertiary carbon structure and stable ether bond makes MTBE resistant to microbial transformation. Initial reports demonstrated biodegradation under aerobic conditions (Salanitro, 1994; Deeb *et al*., 2000; Stocking *et al*., 2000; Fayolle *et al*., 2001) and eventually anaerobic MTBE biodegradation was discovered in several studies under a variety of different redox conditions (Suflita and Mormile, 1993; Mormile *et al*., 1994; Wilson *et al*., 2000; Somsamak *et al*., 2001, 2005, 2006; Bradley *et al*., 2001a, 2002; Finneran and Lovely, 2001; Fischer *et al*., 2005; Pruden *et al*., 2005). Although complete loss and mineralization of MTBE has been demonstrated, MTBE is typically transformed to *tert*-butyl alcohol (TBA), which persists. Utilization of the methyl group can ultimately be coupled to either sulfidogenesis or methanogenesis (Somsamak *et al*. 2001, 2005). However, very little is known about the anaerobic MTBE degradation process and none of the responsible organisms have yet been identified or isolated.

For anaerobic biodegradation to be a reliable method of natural attenuation or enhanced biodegradation of contaminated aquifers, we need more detailed information

about the microorganisms mediating the process. To this end, sediment enrichment cultures supplemented with MTBE as the sole carbon source for over a decade were subjected to molecular community analyses in order to identify the species that are enriched during the degradation process. Previous studies had shown that O-demethylation in these cultures is mediated by bacteria (Youngster *et al*., 2008), so terminal restriction fragment length polymorphism (T-RFLP) analysis in combination with clone analysis was used to identify the bacterial phylotypes enriched on MTBE.

III. Materials and Methods

A. Enrichment cultures and growth conditions

Anaerobic enrichment cultures, originally established from the Arthur Kill Inlet (AK) between New Jersey and New York, Graving Dock (GD) in the San Diego Bay or the New York Harbor (NYH) (Somsamak *et al.*, 2001, 2005), were maintained using strict anaerobic technique under methanogenic or sulfidogenic conditions and were repeatedly transferred, at 6-12 month intervals and usually at a 1:10 dilution, into fresh medium and enriched with MTBE (Aldrich, Milwaukee, WI) as the sole carbon source. Cultures were maintained in 10 to 50 mL glass serum vials capped with Teflon-coated stoppers and aluminum crimp seals and incubated at 28°C. The concentration of MTBE was monitored regularly using gas chromatography with flame ionization detection (Somsamak *et al*., 2001). Select enrichment cultures, representing up to 10^{-7} transfers of the original enrichments, were chosen for DNA extraction and microbial community analysis.

B. DNA extraction and whole genome amplification

DNA was extracted from culture samples using the Power Soil DNA extraction kit (MO BIO, Carlsbad, CA) following the manufacturer's directions. In each sample, DNA was extracted from the community after approximately 50% MTBE degradation. In all samples the quantity of DNA extracted was determined to be insufficient for direct PCR amplification of 16S rRNA genes, thus, whole genome amplification was performed on the extracted DNA samples. This was done using the *illustra* GenomiPhi V2 Genomic amplification kit (GE Healthcare, Piscataway, NJ) following the protocol supplied by the manufacturer for amplification of template DNA. Whole genome amplification increased the DNA concentration 400 to 700-fold, producing sufficient genomic DNA for PCR amplification. The products of whole genome amplification were used for all downstream analyses.

C. 16S rRNA amplification and T-RFLP

PCR with universal bacterial primers, 5'-end 6-FAM labeled 27F (forward) and 519R (reverse), was used to amplify 16S rRNA genes from genomic DNA (Knight *et al.*, 1999). PCR products were digested with *Mnl*I at 37°C for 3 hours. Digested samples were precipitated and resuspended in formamide with a ROX standard and denatured at 95°C (Gallagher *et al*., 2005). Samples were analyzed on a ABI 310 automated sequencer which generated the T-RFLP fingerprint for each community.

D. Cloning, sequencing

A methanogenic enrichment culture established with AK sediment and transferred seven times was selected for clone analysis. The 16S rRNA genes from this culture were amplified by PCR with 27F and 519R primers (Lane, 1991). The PCR products were cloned using the TOPO-TA cloning system (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation. Plasmids were transformed in One Shot TOP10 chemically compentent *Escherichia coli* (Invitrogen, Carlsbad, CA). Transformed cells were plated on LB agar plates containing 75 mg/ml ampicillin with 40µl x-gal per plate. One hundred white colonies were picked and grown overnight in LB- media containing 100 µg/ml ampicillin. Plasmid DNA was extracted from 1 ml of culture using the Purelink Quick Plasmid miniprep kit (Invitrogen, Carlsbad, CA). PCR amplification of the 16S rRNA gene insert was performed using 5'-end 6-FAM labeled 27F and 519R primers and T-RFLP analysis, as described above, to identify unique clones. The complete insert from unique 16S rRNA gene sequences was amplified using M13F and M13R primers. Nucleotide sequencing of unique clones was performed by Genewiz Inc. (North Brunswick, NJ). Rarefaction analysis was conducted using the Analytic Rarefaction software (version 1.3; S.M. Holland, University of Georgia, Athens, GA; http://www.uga.edu/strats/software/Software.html)

E. Phylogenetic analysis

Similar sequences for comparison were found in the Ribosomal Database Project II using the SeqMatch tool (http://rdp.cme.msu.edu). All sequences were aligned using the ClustalX software (Thompson *et al*, 1997). Phylogenetic reconstruction was done

with 309 bp of unambiguously aligned 16S rRNA gene sequence. A tree was constructed with the Phylo_Win program (Galtier *et al*, 1996) using the neighbor-joining method with Jukes-Cantor distances and 1000 bootstrap replications. The final tree was viewed using the NJplot tree drawing program (Perrière & Gouy, 1996). The partial 16S rRNA gene sequences are deposited in GenBank under accession numbers FJ587233 to FJ587239**.**

IV. Results

A. Enrichment on MTBE

Anaerobic sulfidogenic and methogenic enrichment cultures from New York-New Jersey Harbor estuary (AK and NYH) and San Diego Bay (GD) area sediments were maintained on MTBE as the sole carbon source, with repeated transfer, every 6 to 12 months, into fresh medium for a total dilution of 10^{-3} to 10^{-7} of the original culture. MTBE utilization was sustained over this enrichment period. A typical time course of MTBE transformation to *tert*-butyl alcohol is shown in Figure 3.1.

B. T-RFLP community analysis – AK enrichment

Comparative community analysis by T-RFLP provided information on the selection of specific operational taxonomic units (OTU's) after enrichment with MTBE as the sole carbon source. Figure 3.2. shows T-RFLP fingerprints of 16S rRNA genes (following digestion with *Mnl*1), illustrating the change in the methanogenic AK enrichment community following 3, 5 and 7 consecutive transfers. The number of terminal-restriction fragment (T-RF) peaks (OTU's) ranged from over 120 in the 10^{-3}

dilution to 14 in the 10^{-7} dilution. These community profiles after consecutive transfers of the AK sediment enrichment illustrate a decrease in community complexity over time as the culture was enriched on MTBE. The 177, 106 and 206 bp terminal fragments were the only peaks that maintained or increased in relative abundance over time. In the 10^{-7} transfer the predominant phylotypes accounted for 90% of the peak abundance. Particularly notable is the enrichment of the 206 bp fragment from 12% to 64% of the population. The bacterial species corresponding to the 177 and 106 bp T-RFs were stably maintained in the community while the species represented by the 206 bp T-RF significantly increased in prevalence during enrichment with MTBE as the sole carbon source. The abundance of all other bacteria originally present in the MTBE enrichment culture was highly reduced, making up only 10% of the total population after enrichment as estimated from the T-RF areas (if we assume an equal number of 16S rRNA genes and no bias associated with DNA extraction or PCR amplification).

C. Cloning and phylogenetic analysis

To identify the bacteria associated with different T-RFs, a clone library was created, screened and sequenced. Prior to sequencing, T-RFLP analysis of 100 clones from the 10^{-7} AK enrichment culture identified seven unique 16S rRNA phylotypes. The rarefaction curve indicates a low probability of discovering additional phylotypes through continued sampling (Figure 3.3.). T-RFLP fragment lengths for the clones were 106 (35 clones), 128 (5 clones), 177 (22 clones), 206 (25 clones), 237 (8 clones), 250 (4 clones), and 293 bp (1 clones), respectively. The T-RFLP fingerprints of the individual clones matched all the predominant terminal fragments of the community fingerprints of the AK

enrichment culture, indicating that all predominant phylotypes of the culture were identified.

The phylogenetic tree (Figure 3.4.) compares the clones to the closest cultured organisms and selected known O-demethylating bacteria and illustrates that the clones span several phyla. The 206 bp clone clusters with members of *Chloroflexi*, the 106 bp clone with *Firmicutes*, the 177 bp clone with *Deltaproteobacteria*, the 250 and 128 bp clones with *Thermotogae*, the 237 bp clone with *Alphaproteobacteria*, and the 293 bp clone with *Bacteroidetes*. Comparison of T-RFLP fingerprints after three, five and seven consecutive transfers indicate that enrichment on MTBE was leading to a prevalence of organisms of the phyla *Firmicutes*, *Deltaproteobacteria* and *Chloroflexi*. The closest described species to the *Firmicutes* clone is *Anaerococcus prevotii*, with 95% homology of 516 bp compared. The closest described species to the *Deltaproteobacteria* clone is *Geobacter metallireducens*, with 90% homology when 540 bp are compared. The *Chloroflexi* clone did not show close homology with any cultured species in the phylum. This clone had 79% similarity to *Dehalococcoides ethenogenes* of 488 bp compared. The closest 16S rRNA sequence found was that of an uncultured salt marsh bacterium (Genbank accession AY711255), which shared 96% similarity with the clone when 416 bp were compared.

D. Comparison of bacterial communities enriched on MTBE – AK, GD and NYH sediments

Interestingly, the T-RFLP profiles of the methanogenic enrichments established from GD, AK and NYH sediments all displayed substantially different major T-RF peaks

(Figure 3.5.). There were few peaks in common and no site shared predominant peaks at comparable levels of dilution. All three communities shared a peak at 177 bp, however this T-RF was only a very minor component of the GD profile. GD shared a 206 bp peak (*Chloroflexi*) with the AK community, however this T-RF was absent in the NYH profile. The NYH community shared a 106 bp peak (*Firmicutes*) with the AK community, but this T-RF was quite minor in the NYH profile and absent from GD. Figure 3.6. shows the comparison between corresponding transfers of methanogenic and sulfidogenic cultures from GD and NYH. A high degree of similarity was seen between the sulfidogenic and methanogenic enrichment cultures of each site, with the NYH cultures being almost identical to each other. This suggests that the electron accepting condition did not substantially influence the bacterial communities that were enriched on MTBE.

V. Discussion

Anoxic conditions are common in gasoline polluted groundwater, therefore anaerobic MTBE degradation is potentially an important means of remediation (McMahon *et al*., 2008). Although anaerobic degradation of MTBE is now well established, many studies attempting to promote anaerobic MTBE degradation have yielded negative results or observed degradation in only a few of many established cultures (Suflita and Mormile, 1993; Borden *et al*., 1997; Somsamak *et al*., 2001; Chen *et al*., 2005; Fischer *et al*., 2005; Martienssen *et al*., 2006). This suggests that the organisms responsible for anaerobic MTBE degradation or the conditions necessary for the process are sparsely distributed, even in contaminated soils and sediments. Information about the

organisms involved in anaerobic MTBE degradation is critical for understanding this process and will be useful for monitoring and assessment of contaminated aquifers.

Here we present characterization of the bacterial communities of anaerobic MTBE degrading cultures enriched from different sediments of the New York-New Jersey Harbor estuary and San Diego Bay (Somsamak *et al*., 2001, 2005). Characteristics of the cultures suggest that acetogenic bacteria mediate the initial ether cleavage and utilization of the methyl group. Propyl iodide caused light-reversible inhibition of MTBE-degradation, suggesting that the MTBE degradation process was corrinoid-dependent (Youngster *et al*., 2008; Chapter 2). In addition, inhibitors of methanogenesis or sulfidogenesis did not completely block anaerobic MTBE utilization (Somsamak *et al*., 2005). Methanogens or sulfidogens may, however, facilitate MTBE degradation perhaps by utilizing the products of the acetogenic metabolism, and overall carbon flow in these cultures was eventually coupled to methangenesis or sulfate reduction, respectively (Somsamak *et al*., 2001, 2006). Characterization of the MTBE utilizing enrichment cultures is hampered by their very slow growth. Cultures could be transferred with a 1:10 dilution, at most, only every 6 to 12 months. Thus, even after close to 10 years of enrichment, cultures have only been diluted to 10^{-7} of the original. Furthermore, the population density is very low, necessitating an initial whole genome amplification of the DNA prior to PCR. Community analysis focused on the bacterial population because previous studies had shown that rifampicin, a bacterial protein synthesis inhibitor, prevented anaerobic MTBE degradation, indicating that the initial attack on MTBE is mediated by bacteria, not archaea (Youngster *et al.*, 2008; Chapter 2). The sulfidogenic and methanogenic enrichment cultures enriched from the one site had a very similar

population structure, suggesting that the electron accepting condition did not substantially influence the bacterial communities that were enriched on MTBE.

T-RFLP fingerprints of the anaerobic enrichment cultures after several transfers revealed how enrichment on MTBE reduced the complexity of the microbial community. From the AK sediment, three dominant phylotypes were enriched and, as expected, the extent of purification suggests that one or more of these mediates the initial attack on MTBE and uses the methyl group as a carbon source. These clustered with the phylum *Deltaproteobacteria* (177 bp clone), the *Chloroflexi* (206 bp clone) and *Firmicutes* (106 bp clone). Members of the *Deltaproteobacteria* are well known for fermentation of different substrates (Kersters *et al*., 2006). The *Firmicutes* contains several organisms that are known to O-demethylate aryl-methyl ethers, such as *Acetobacterium woodii, Eubacterium limosum,* and *Syntrophococcus sucromutans* (Frazer, 1994). However, the *Firmicutes* clone discovered in this study was very distantly related to known members of the phyla, with 77-79% similarity to 16s rRNA genes from these organisms. The *Chloroflexi*, which contains the clone that is most heavily selected for by enrichment on MTBE, is a metabolically diverse group with representatives widely distributed in the environment (Hanada and Pierson, 2006). Based on phylogeny, we are thus unable to conclusively determine which of these organisms is responsible for MTBE degradation in the enrichment cultures, or if all three are responsible. Knowledge of the predominant markers should allow for targeting the enrichment and selection of the MTBE-degrading organism(s).

The continued presence of these multiple T-RF peaks, even after several years with MTBE as the sole carbon source and seven transfers, indicates that inter-species

interactions of a consortium may be required for appreciable anaerobic MTBE degradation to occur. Symbiotic relationships, where some species remove the transformation products created by the breakdown of an initial substrate, can improve the energetic efficiency of microbial degradation reactions. If acetogenesis is coupled to the anaerobic O-demethylation of MTBE, we expect that one species mediates the initial O-demethylation and acetogenesis and that acetate is then consumed by other members of the community. However, the lack of common predominant peaks in T-RFLP fingerprints of the different sediment (AK, NYH, and GD) enrichment cultures indicate that there are likely several different MTBE-degrading organisms present at the different sites.

Ultimately, a comprehensive understanding of the different microbial populations and how their activities can be enhanced is important for optimizing biodegradation conditions and identifying amendments that stimulate anaerobic MTBE degradation *in situ*. Identification of the microorganisms mediating anaerobic MTBE degradation should provide bioindicators for monitoring natural or enhanced *in situ* biodegradation in polluted environments. Molecular monitoring of the bacterial population responsible for MTBE degradation can also be used in combination with stable isotope analysis (Hunkeler *et al*., 2001; Kolhatkar *et al*., 2002; Somsamak *et al*, 2005, 2006; Zwank *et al*., 2005; McKelvie *et al*., 2007; and Busch-Harris *et al*., 2008) for site assessment. This research is important for gaining an understanding of different microbial processes and how these processes, and thus the remediation, are affected by different amendments and other engineering approaches.

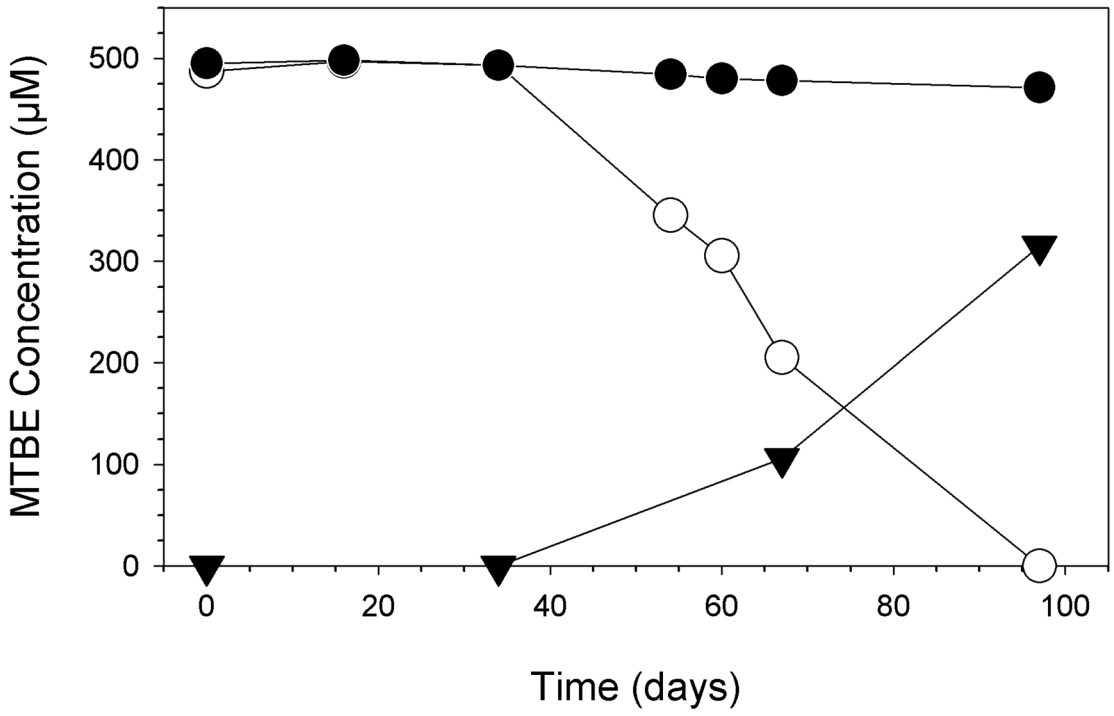


FIGURE 3.1. MTBE (○) utilization and TBA (▼) accumulation in an anaerobic 7th transfer methanogenic AK enrichment culture. No MTBE (●) loss in the abiotic control.

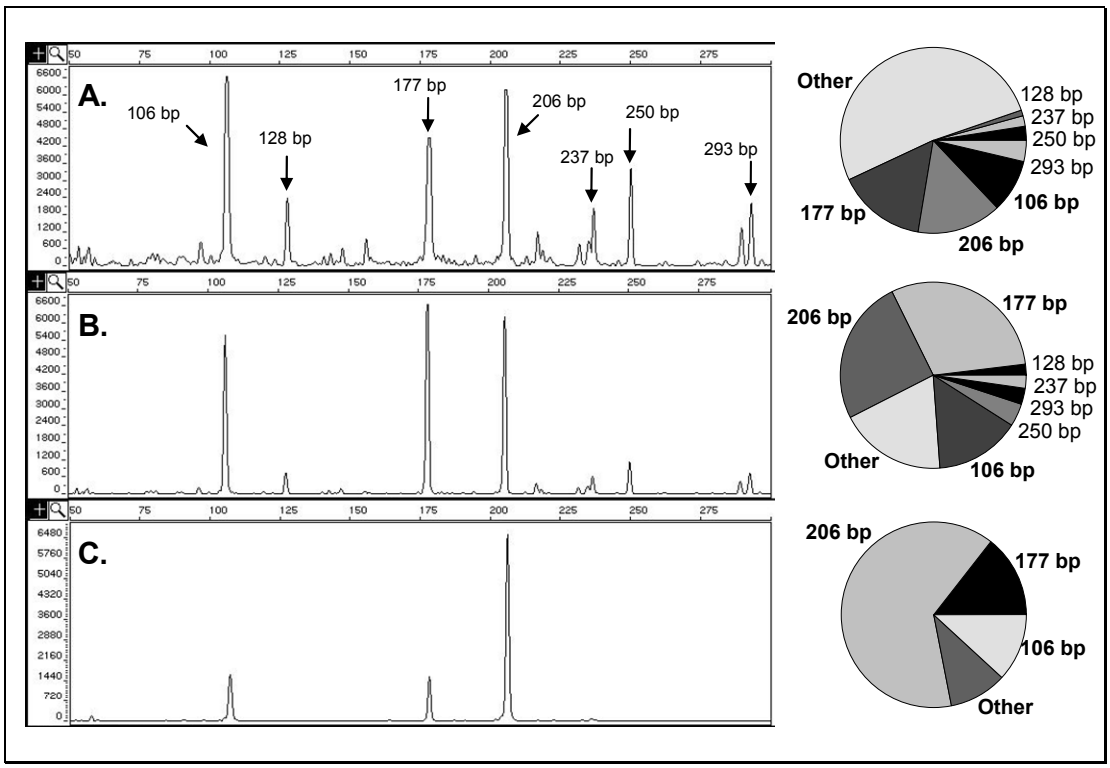


FIGURE 3.2. T-RFLP analysis of MTBE utilizing methanogenic enrichment cultures of AK sediment after sequential enrichments and transfers to 10^{-3} (A), 10^{-5}(B) and 10^{-7} (C) of the original culture. Enrichments were transferred at 6 to 12 month intervals and fed MTBE 3 to 4 times between transfers. Pie charts to the right indicate the relative abundance of individual terminal restriction fragments.

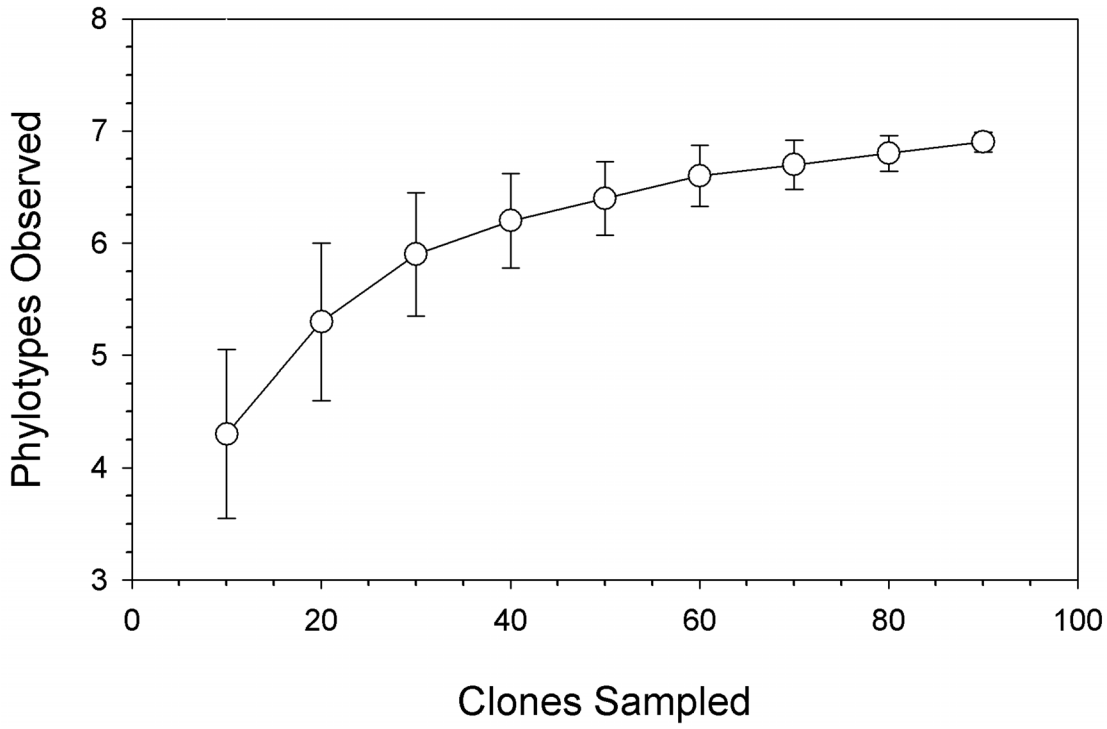


FIGURE 3.3. Rarefaction analysis. Indicates probable numbers of phylotypes to be found (y axis) when a given number of clones are analyzed (x axis) from the methanogenic 10^{-7} AK enrichment culture.

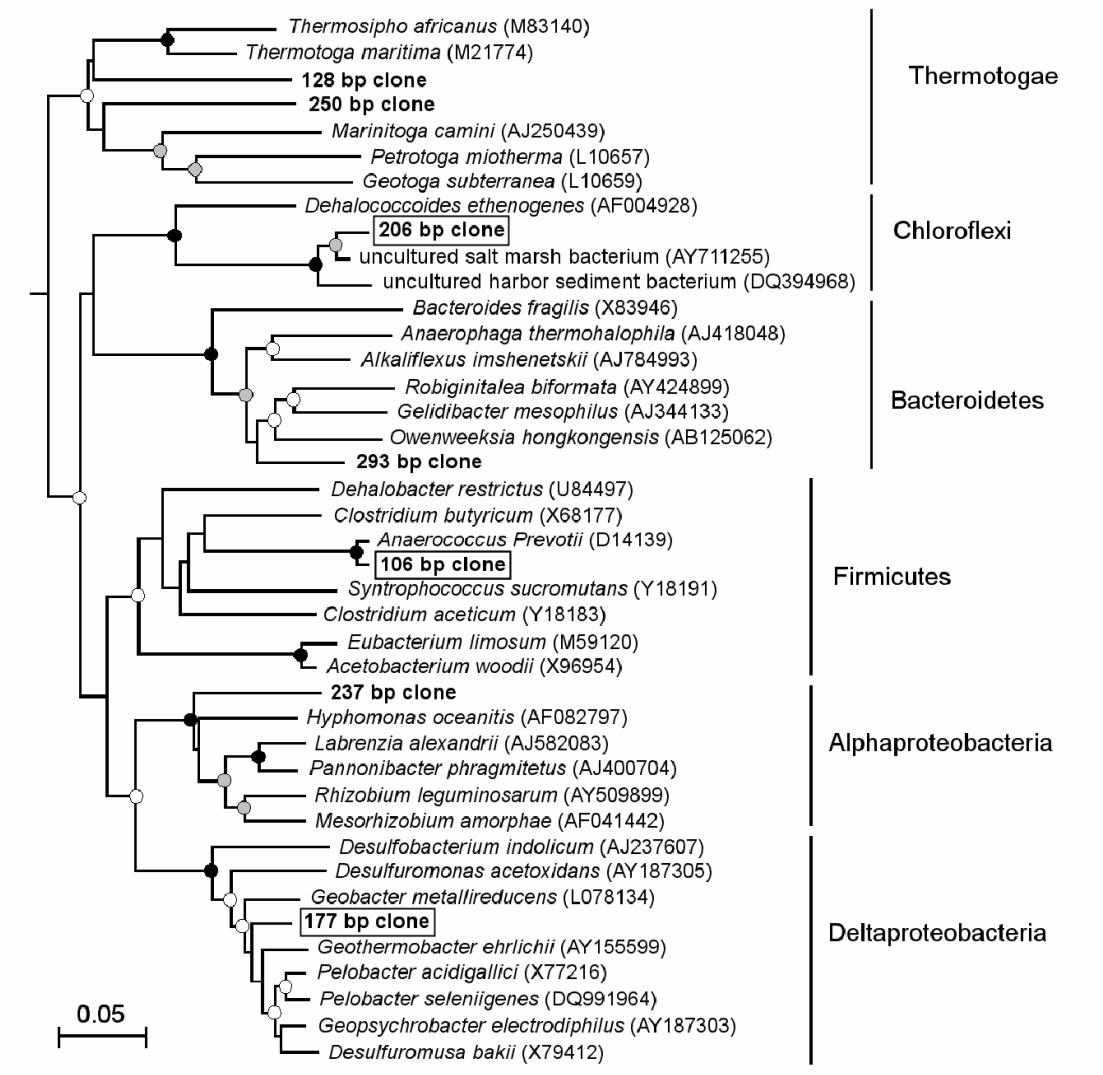


FIGURE 3.4. Neighbor joining phylogenetic tree based on partial bacterial 16S rRNA gene sequences cloned from a 10^{-7} MTBE degrading enrichment culture of AK sediment. *Halobacterium salinarum* was used as an outgroup. A 309 bp gene fragment was used for analysis. Bootstrap values are indicated by greyscaled nodes; black: >90, gray >70, white >50. The scale bar indicates expected number of nucleotide substitutions per site per unit of branch length.

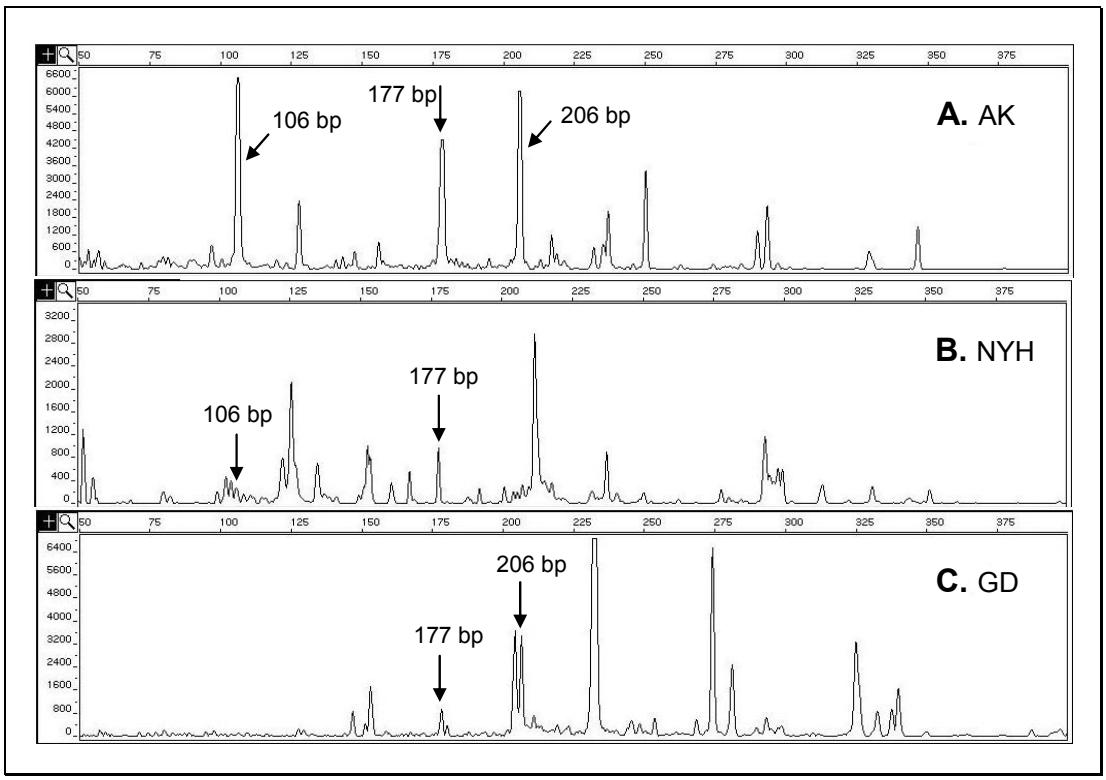


FIGURE 3.5. T-RFLP analysis of 10^{-3} dilutions of methanogenic MTBE degrading enrichment cultures established from AK (A), NYH (B) and GD (C) sediments.

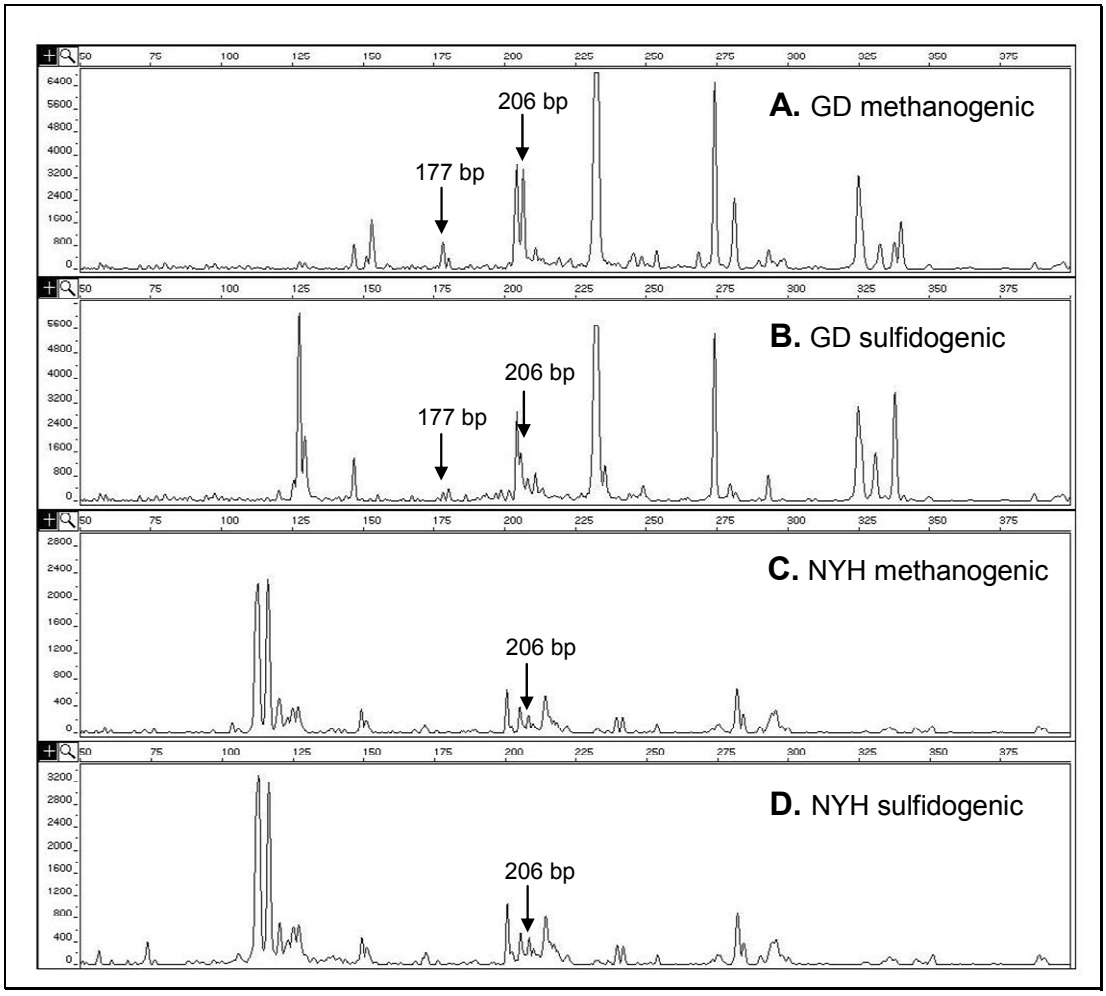


FIGURE 3.6. T-RFLP analysis of MTBE degrading enrichment cultures (10^{-3})of GD (A and B) and NYH (C and D) sediments maintained under methanogenic and sulfidogenic conditions, respectively.

Chapter 4

Stable isotope probing of anaerobic methyl *tert*-butyl ether (MTBE) degrading enrichment cultures

I. Abstract

Groundwater contamination with methyl *tert*-butyl ether (MTBE) is an extensive problem in the United States and other industrialized nations where the chemical has been used as a fuel oxygenate. Microbial mediated natural attenuation is an attractive option for remediation, however there is insufficient information about MTBE biodegradation processes, particularly in anaerobic environments. Serial dilution of sediment enrichment cultures has failed to yield pure cultures nor conclusive identification of the MTBE degrading organisms, therefore emphasis needs to be placed on studying MTBE degrading communities via culture-independent methods. Stable isotope probing (SIP) allows for the tracking of stable isotope markers from labeled substrates into cellular biomarkers to identify the organisms responsible for degradation of these compounds. Here we report the results of the first SIP experiments with anaerobic MTBE degrading enrichment cultures. SIP was combined with terminal restriction fragment length polymorphism (T-RFLP) analysis of the 16S rRNA genes in an enrichment of sediment from the New York Harbor. Results show the sequential incorporation of the ^{13}C label of MTBE by the bacterial community, indicating that SIP may be an excellent technique for identification of the organisms that are the first to utilize the carbon from the O-methyl group of MTBE in these enrichment cultures.

II. Introduction

In 1999, U. S. production of methyl *tert*-butyl ether (MTBE) peaked at over 9,200 million kg/year (US EPA, 1999; EIA/DEO, Häggblom *et al*., 2007). An inexpensive synthetic chemical formed by the reaction of methanol with isobutylene, MTBE was added to fuel at up to 15% by volume to increase octane and reduce hazardous combustion emissions (Franklin *et al*., 2000; US EPA, 2000). Today, MTBE use in gasoline is banned or restricted in most of the United States. However, over the course of over a decade of heavy use, MTBE emerged as a frequently detected and extremely resilient groundwater contaminant (Squillace *et al*., 1996, 1999; Pankow *et al*., 1997; Reuter *et al*., 1998; Dernbach, 2000; Johnson *et al*., 2000; Achten *et al*., 2002a, 2002b; Toran *et al*., 2003; Heald *et al*., 2005 Ayotte *et al*., 2005, 2008). Despite drastically decreased production and use, MTBE contamination persists because of unfortunate physical and chemical properties that allow the compound to withstand many common methods used to remediate polluted water resources (Squillace *et al*., 1997; Stocking *et al*., 2000).

Biotransformation is an advantageous method of removal as it permanently eliminates MTBE from the ecosphere, however, we need a better understanding of the process to be able to optimize microbial mediated natural attenuation under environmental conditions. Although aerobic MTBE degrading organisms have been isolated and can be cultured for use in bioreactors for assisted bioremediation (Wilson, 2003), many MTBE contaminated water sources are predominantly anoxic. While anaerobic MTBE degradation occurs, little is known about the process and the responsible organisms are unisolated. Anaerobic MTBE biodegradation has been

detected in laboratory microcosm and field studies (Mormile *et al*., 1994; Yeh and Novak, 1994; Wilson *et al*., 2000; Somsamak *et al*., 2001, 2005, 2006; Bradley *et al*., 2001a, 2002; Finneran and Lovely, 2001; Fischer *et al*., 2005; Pruden *et al*., 2005). We have enriched anaerobic MTBE degrading cultures on MTBE for many years and partially characterized these communities using molecular and cultural techniques (Youngster *et al*., 2008; Chapter 2 and 3). 16S rRNA genes from a highly enriched community have been cloned, sequenced, and mapped to their nearest phylogenetic relatives, however this does not reveal which organisms are actively O-demethylating MTBE and using the methyl group as a carbon source. Terminal-restriction fragment length polymorphism (T-RFLP) analyses have revealed the sustained presence of multiple species in laboratory cultures that have been enriched on MTBE as the sole carbon source for multiple transfers (Chapter 3), suggesting widespread cross-feeding or possibly that microbial consortial interactions may be required for anaerobic MTBE biodegradation.

Stable isotope probing (SIP) is a culture-independent technique for examining complex microbial populations which can be used to link microbial identitification with metabolic activity within the community (For reviews, see: Radajewski *et al*., 2000, 2003; Wellington *et al*., 2003; Manefield *et al*., 2004; Wackett, 2004; Dumont and Murrell, 2005; Neufeld *et al*., 2006; Kreuzer-Martin, 2007; Neufeld *et al*., 2007; Kerkhof and Häggblom, 2008). In SIP experiments, a stable isotope labeled substrate is fed to the community. If microbes within the community actively catabolize this substrate, the stable isotope label is incorporated into their nucleic acids, fatty acids, and proteins which are subsequently extracted to be analyzed by molecular community characterization

techniques. Separate analysis of the extracted molecular species with and without incorporated isotopic labels enables identification of members of the community that utilize and assimilate the substrate of interest.

In this study, anaerobic sediment enrichment cultures that have maintained MTBE degradation activity for over a decade were fed ^{13}C-labeled MTBE for SIP analysis. ^{13}C-labeled DNA was isolated and 16S rRNA genes were amplified from this labeled DNA for further characterization by T-RFLP analysis. Comparison of community profiles of ^{13}C labeled DNA versus the entire community identifies which community members are the first to use the carbon from MTBE.

III. Materials and Methods

A. Cultures and growth conditions

Anaerobic MTBE degrading enrichment cultures were previously established with sediments collected from various sites and stored at 4^{o}C (Somsamak *et al.*, 2001, 2005). Cultures originating from the New York Harbor (NYH) were maintained using strict anaerobic technique under methanogenic conditions and were repeatedly transferred into fresh medium at 6-12 month intervals, and enriched with MTBE (Aldrich, Milwaukee, WI) as the sole carbon source. Six replicate enrichment cultures, representing 10^{-7} transfers of the original enrichments, were chosen for SIP experiments. These cultures were transferred to 150 mL glass serum vials, capped with Teflon-coated stoppers and aluminum crimp seals and incubated at 28°C. Cultures were spiked with 300 μM MTBE with ^{13}C labeled O-methyl carbon (provided by Dr. Richnow, UFZ, Leipzig). Control cultures were spiked with 300 μM ^{12}C-MTBE. MTBE concentration was regularly

monitored using gas chromatography with flame ionization detection (Somsamak *et al*., 2001). At 6 weekly timepoints, 6-10 ml samples were withdrawn and sacrificed from the enrichment cultures for DNA extraction and further analyses.

Cultures of the archaeon, *Halobacterium salinarium,* were grown in either marine broth 2216 (Difco, Detroit, Michigan) or ^{13}C-labeled ISOGRO powder growth medium (Isotec, Miamisburg, OH), diluted 1:3 with sterile 4.5 M NaCl. Cultures were incubated at 37°C with shaking until they reached an appropriate density for harvesting and DNA extraction.

B. DNA extraction and separation on CsCl gradient

DNA was extracted from enrichment culture and *H. salinarium* cultures using either the Power Soil DNA extraction kit (MO BIO, Carlsbad, CA) following the manufacturer's directions or a modified phenol-chloroform extraction method (Scala and Kerkhof, 2000). Extracted DNA samples were resuspended in 30 μL sterile water. This entire 30 μL of resuspended DNA, as well as approximately 300 ng each of ^{12}C- and ^{13}C-labeled *H. salinarium* DNA, were added to a 500 μL CsCl density gradient (~1 g/mL) containing ethidium bromide. ^{12}C- and ^{13}C-labeled DNA were separated by 36 hours of centrifugation at 80,000 rpm on a Beckman Optima ultracentrifuge (Palo Alto, CA) using a TLA 120 rotor (Gallagher *et al*., 2005). Bands were withdrawn from the CsCl gradient using UV light visualization and samples were dialyzed (in 0.4M Tris) for one hour (Gallagher *et al*., 2005).

C. DNA amplification, purification and T-RFLP analysis

Dialyzed DNA samples were subjected to whole-genome amplification using the *illustra* GenomiPhi V2 Genomic amplification kit (GE Healthcare, Piscataway, NJ), following the manufacturer's protocol for amplification of template DNA. 16S rRNA genes were then amplified by PCR from this genomic DNA using universal bacterial primers, 5'-end 6-FAM labeled 27 (forward) and 519 (reverse) (Knight *et al.*,1999). 50 µL PCR reactions were often run in triplicate and the reactions were pooled, precipitated using sodium acetate and ethanol and resuspended in a smaller volume (30 µL) for agarose gel purification. Agarose gel purification was performed using the E.Z.N.A gel extraction kit (Omega Bio-Tek, Frederick, CO), DNA was eluted from the filter twice in 50 µL of sterile deionized water and then concentrated in a smaller volume (10 µL) for T-RFLP analysis. Purified PCR products were prepared for T-RFLP with a 6 hour digestion with *Mnl*I at 37°C, followed by precipitation with sodium acetate and resuspension in formamide with a ROX standard (Gallagher *et al*., 2005). Samples were denatured at 95°C and analyzed on an ABI 310 automated sequencer, which produced a T-RFLP community fingerprint for each sample.

IV. Results

MTBE utilization was sustained for several years of enrichment in anaerobic methanogenic enrichment cultures from New York Harbor sediments. Cultures were repeatedly transferred at 6-12 month intervals into fresh medium and given MTBE as the sole carbon source for a total dilution of 10^{-7} of the original culture at which point they

were amended with ^{13}C-labeled MTBE for SIP experiments. Degradation of ^{13}C-labeled MTBE proceeded more slowly than typical for degradation of ^{12}C-MTBE (Figure 4.1.). To identify members of the enrichment culture community that actively incorporated the ^{13}C label into their DNA, ^{13}C-enriched DNA molecules were separated from ^{12}C DNA by density gradient centrifugation. DNA was extracted from enrichment culture samples at multiple timepoints and the samples were centrifuged in a cesium chloride gradient to separate ^{13}C DNA from ^{12}C. Following the protocol developed by Gallagher *et al*. (2005), unlabeled and ^{13}C labeled *H. salinarium* carrier DNA were added to each CsCl gradient to enable visualization of both bands following centrifugation. Previous testing of ^{12}C and ^{13}C *H. salinarium* DNA, where the DNA was added to a CsCl gradient, centrifuged, extracted, and PCR amplified using 16S rRNA primers indicated that the *H. salinarium* DNA was free from contamination of any bacterial DNA that could interfere with sample analysis. Control cultures that were fed ^{12}C-MTBE were processed identically to ^{13}C -MTBE experimental samples. When ^{13}C bands, isolated from ^{12}C-MTBE control DNA, were used as template DNA, no 16S rRNA gene amplification was detected, indicating the presence of only the archaeal DNA. This control also indicated that the ^{13}C bands of the ^{13}C -MTBE treated samples were also free of susbstantial ^{12}C DNA contamination

At least 6 timepoint samples each from 6 different ^{13}C-MTBE treated cultures were processed. Of these 36 extracted DNA samples, only three successfully yielded PCR amplified 16S rRNA product, suitable for T-RFLP analysis, from both the ^{13}C and ^{12}C bands following CsCl gradient centrifugation. Two of the samples were from the same NYH enrichment culture (NYH1) at different MTBE degradation timepoints. The

third sample was from a second NYH enrichment culture (NYH2). Of the NYH1 samples, the first timepoint sample was taken after 21 days incubation. The community fingerprint from the unlabeled DNA fraction shows high diversity (42 detectable T-RFs), with predominant T-RFs at 168, 210, 250, 273 and 277 bp (Figure 4.2.). The fingerprint from the ^{13}C labeled fraction of DNA had lower diversity, with only 22 detectable T-RFs. In the ^{13}C labeled community profile the 131, 168, 210 and 291 bp T-RFs dominated the profile, while T-RFs at 250, 273 and 277 were not present or present at extremely low levels. The presence of fewer T-RFs in the ^{13}C labeled DNA band, suggests that these community members are the foremost consumers of the added ^{13}C-MTBE. After 35 days incubation, the profiles from ^{13}C labeled DNA and from the unlabeled DNA band were highly similar, suggesting that the ^{13}C from MTBE has been universally distributed throughout the population at this timepoint.

Only one timepoint was successfully analyzed for sample NYH2. The DNA was collected after 28 days incubation. It is an informative timepoint to study, as there are differences between the community fingerprint from the unlabeled DNA fraction and the ^{13}C labeled fraction (Figure 4.3.). Both DNA samples show high diversity, with 54 and 52 OTUs, respectively. However the unlabeled DNA profile indicates a presence of 210, 250, 273 and 277 bp T-RFs, which are not present in the ^{13}C labeled DNA profile. In the ^{13}C labeled community profile, 139, 168, 213 and 293 bp T-RFs dominate the profile, suggesting that these organisms are the primary consumers of the ^{13}C labeled MTBE in this community.

V. Discussion

This study is the first to use SIP to examine anaerobic MTBE degrading enrichment cultures and identify the species assimilating the methyl group of MTBE. We observed slower degradation of ^{13}C-labeled MTBE than that of ^{12}C-labelled MTBE. This was expected since compound specific isotope analysis studies showed substantial carbon isotope fractionation during anaerobic MTBE degradation (Somsamak *et al*., 2005, 2006), suggesting that increased kinetic energy is required for O-demethylation of ^{13}C-MTBE (Rosell *et al*., 2007). Comparison of community profiles generated at different degradation timepoints in this study indicate that some members of the community assimilate ^{13}C atoms from the labeled MTBE substrate into their DNA before others. Presumably, one or more of the organisms present in the ^{13}C labeled community profile are the primary O-demethylating organisms of the community. Each of the three ^{13}C labeled community profiles featured a major T-RF of 168 bp, suggesting the possibility that the species represented by this peak is responsible for O-demethylation of MTBE in both NYH1 and NYH2 cultures. Future experiments will include construction and sequencing of a clone library of this community to determine the phylogenetic identity of this organism and others that are ^{13}C labeled at the earlier examined timepoint in culture NYH1.

Previous T-RFLP and clone sequence analysis of an anaerobic MTBE degrading enrichment culture initiated from a different sediment source (Arthur Kill Inlet, New York/New Jersey) revealed organisms from three phyla that were preferentially enriched over successive transfers (Chapter 3). Although the T-RFs are different between that study and the present work, it will be interesting to see if the sequences of the ^{13}C-

incorporating organisms in this SIP experiment are closely related to the sequenced clones from the previous study. As found in prior phylogenetic characterizations of MTBE degrading enrichment cultures, this study detected the sustained presence of multiple T-RFs in the community even after a lengthy period of sustained enrichment on MTBE as the sole carbon source. The SIP results strongly indicate that substantial cross-feeding occurred in the NYH1 enrichment culture community, as the ^{13}C label became distributed throughout the community over time. It is also possible that there are multiple organisms in the consortium capable of the initial O-demethylation of MTBE.

The results presented here indicate that DNA-SIP will be an effective method for studying the carbon flow in anaerobic MTBE degradation and for identifying MTBE O-demethylating organisms. This is an important finding since there are multiple approaches to SIP experiments which do not all work equally well under different conditions and for different substrates. As pointed out by Neufeld *et al*. (2006), when one examines articles about SIP, there is a high ratio of reviews to primary publications, a situation reflective of the challenges of the technique. The different cellular biomarkers that can be monitored during a SIP experiment (DNA, RNA, proteins and fatty acids) differ in the information that they can provide about the parent organism and in their sensitivity. DNA-SIP links functional activity within the community to phylogenetic marker genes, such as 16S rRNA. However, separation of ^{13}C and ^{12}C DNA requires high incorporation of ^{13}C (at least 15-20%) which is slow for DNA, reducing sensitivity of the experiment (Radajewski *et al*., 2000). Therefore, DNA-SIP is best-suited for determining carbon flow in communities where the initial consumers only have ^{13}C labeled carbon available for catabolism. The highly enriched anaerobic enrichment

cultures used in this study are good candidates for SIP analysis because MTBE is the only provided carbon source and thus, most of the carbon flow in the community should be initiated from MTBE O-demethylation. This study confirms that the isotopic incorporation of ^{13}C into DNA of organisms in the anaerobic MTBE degrading enrichment cultures was sufficient for detection of ^{13}C labeled DNA in our extracted sample DNA.

The current study highlighted concerns that will need to be addressed in future experimental designs. DNA extractions were performed on 36 timepoint samples, however a PCR product suitable for T-RFLP analysis was only obtained from three of these. Sample loss occurred during the processing steps between DNA extraction and T-RFLP analysis. Some of this problem can be resolved by setting up several replicates of all whole genome amplification and PCR reactions and pooling the products. We recommend that as an additional measure in preparation for future SIP experiments, larger volume enrichment cultures must be started to enable sacrifice of greater biomass amount for DNA extraction at more frequent timepoints. DNA extraction from a larger volume of culture will provide more initial DNA to work with, resulting in a higher proportion of samples producing usable quantities of DNA for PCR amplification and cloning. Sampling at additional timepoints will provide more detailed information about the carbon flow in the community and improve the probability of sampling at a stage of degradation which will provide identification of the very first organisms that incorporate the ^{13}C label from MTBE.

Understanding the microbial populations that mediate anaerobic MTBE degradation will be important for optimizing anaerobic biodegradation conditions *in situ*.

Identifying biomarkers for anaerobic MTBE degrading microorganisms will be a key to understanding why some enrichment cultures have biodegradation activity and not others, even amongst those established at the same time from the same sediment location. Biomarker identification for MTBE O-demethylators will also allow prediction of the biodegradation capabilities of bacterial communities in polluted environments as well as assessment of the performance of assisted natural remediation procedures in anaerobic environments.

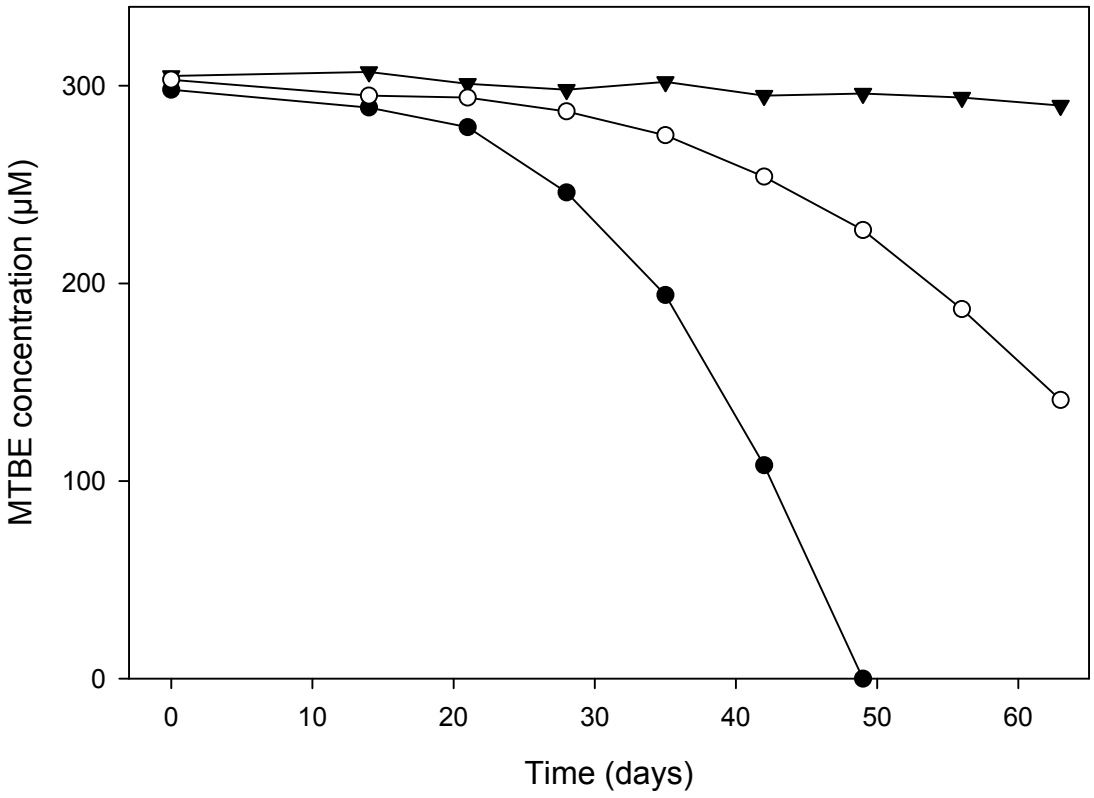


FIGURE 4.1. Utilization of ^{12}C-MTBE (●) and ^{13}C-MTBE (○) in anaerobic methanogenic NYH enrichment cultures. No ^{13}C-MTBE (▲) loss in the abiotic control.

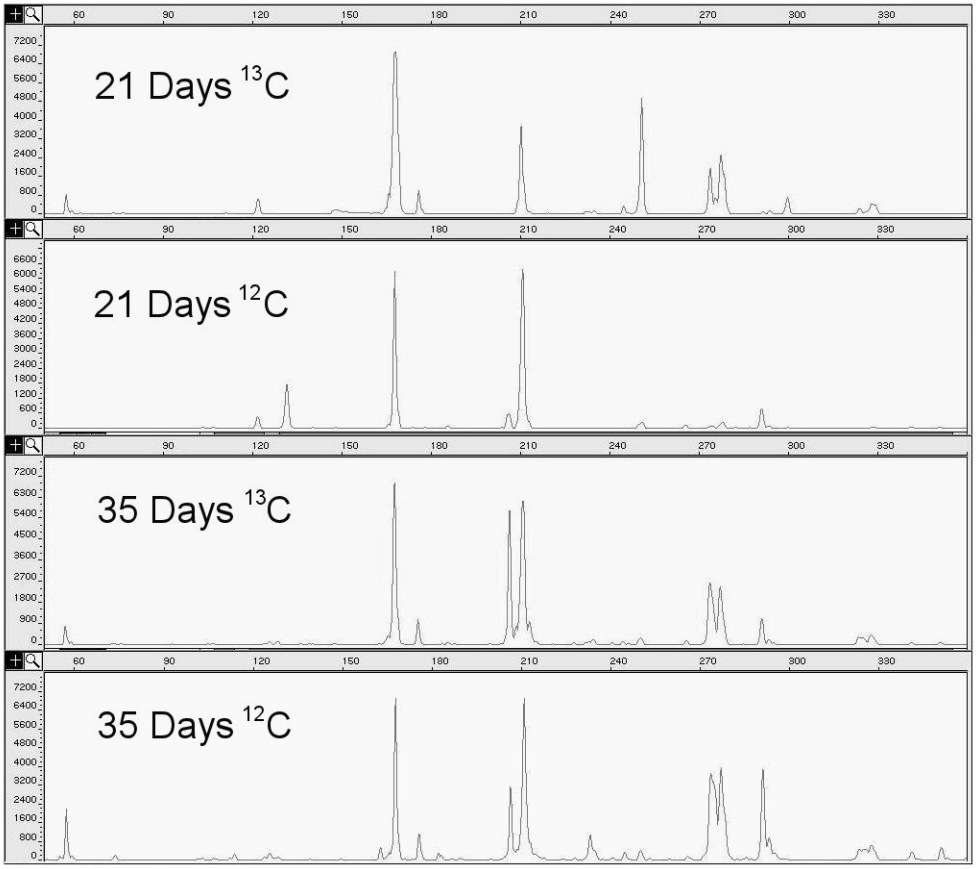


FIGURE 4.2. 16S rRNA gene T-RFLP analysis of NYH1 MTBE-utilizing methanogenic enrichment cultures at two different MTBE degradation timepoints (21 and 35 days), showing communities from the ^{12}C and ^{13}C bands following density-based DNA separation.

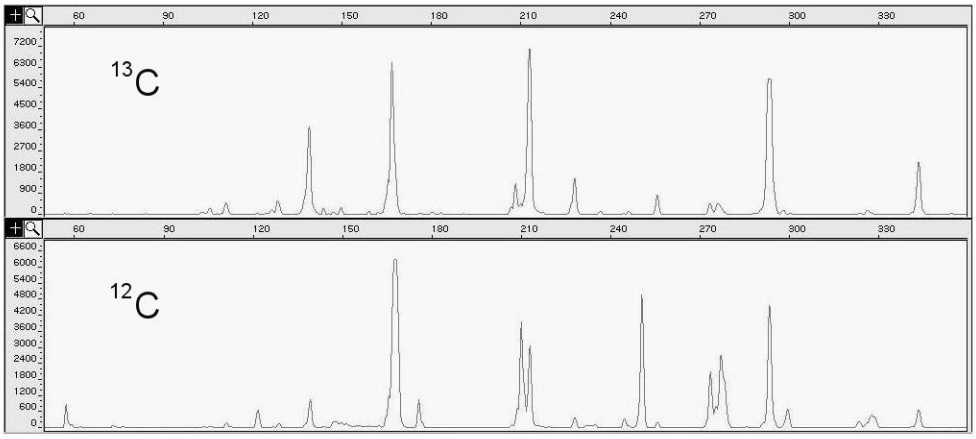


FIGURE 4.3. 16S rRNA gene T-RFLP analysis of NYH2 MTBE-utilizing methanogenic enrichment culture at 28 days degradation, showing communities from the ^{12}C and ^{13}C bands following density-based DNA separation.

Chapter 5

Carbon and hydrogen isotope fractionation during methyl *tert*-butyl ether biodegradation

I. Abstract

The fuel oxygenate, methyl *tert*-butyl ether (MTBE), although now widely banned, remains a persistent groundwater contaminant. Biodegradation is a promising means for remediating contaminated aquifers, however site assessment and *in situ* bioremediation monitoring present several challenges. Compound specific isotope analysis (CSIA) is being developed as a tool for determining the extent of MTBE loss due to biodegradation. Multidimensional CSIA of carbon and hydrogen can potentially not only distinguish between compound loss due to biodegradation and physical processes, but also between different types of biodegradation processes. In this study, carbon and hydrogen isotopic fractionation factors were determined for MTBE degradation in aerobic and anaerobic laboratory cultures under a variety of conditions. Carbon isotopic enrichment factors for aerobic MTBE degradation by a bacterial consortium containing the aerobic MTBE-degrading bacterium, *Variovorax paradoxus,* were -1.13 ± 0.19‰ and hydrogen isotope enrichment factors were -14.58 ± 2.06‰. Incubation temperature did not affect carbon or hydrogen isotope fractionation during aerobic MTBE degradation. Carbon isotope enrichment factors for anaerobic MTBE degrading enrichment cultures were -6.95 ± 0.19‰. Carbon fractionation during anaerobic MTBE degradation did not vary based on the collection site of the original sediment, redox condition of the enrichment, or supplementation with syringic acid as a co-substrate. The hydrogen enrichment factors of anaerobic enrichment cultures without syringic acid was insignificant, however a strong average hydrogen enrichment factor of -41.11 ± 2.66‰ was found for cultures which were fed syringic acid as a co-substrate during anaerobic MTBE degradation.

II. Introduction

Methyl *tert*-butyl ether (MTBE) was introduced in the late 1970s as a fuel octane enhancer and was used extensively throughout the 1990s as a fuel oxygenate in reformulated gasoline to comply with the U.S. Clean Air Act amendments of 1990 (Franklin *et al*., 2000). United States production of MTBE peaked at over 9,200 million kg/year in 1999 (EIA/DEO; Häggblom *et al*., 2007). Production has tapered off in recent years as many U.S. states have banned or restricted MTBE use in fuel due to widespread groundwater contamination. MTBE removal is expected to be costly, with current estimates ranging from $4 to 85 billion for cleanup of MTBE contaminated public U.S. water supplies (AWWA, 2005). The physical and chemical properties of MTBE, including high water solubility, make it a persistent contaminant, resistant to many common fuel spill remediation techniques (Squillace *et al*., 1997; Stocking *et al*., 2000; US EPA, 2000). Natural attenuation through microbial degradation has been observed under a variety of environmental conditions (for reviews see: Squillace *et al*., 1997; Deeb *et al*., 2000; Stocking *et al*., 2000;Häggblom *et al*., 2007) and is a potentially important and affordable means for remediation of contaminated aquifer.

For natural attenuation to be reliable, it is crucial to be able to determine if and at what rate biodegradation is occurring *in situ*. Monitoring concentration changes in environmental samples is inadequate to conclude if any decrease is due to biodegradation or other processes. Volatilization, dilution, and sorption to sediment may lower the concentration of MTBE in an aquifer, however, biodegradation is the only method of natural attenuation that results in a lasting removal of MTBE from the ecosystem. Monitoring *in situ* MTBE biodegradation is additionally complicated in that *tert*-butyl

alcohol (TBA) is a primary biotransformation product, but also a likely fuel component and environmental co-contaminant at MTBE contaminated locations (Landmeyer *et al*., 1998). This makes it impractical to assess *in situ* biodegradation activity by measuring the concentration of biotransformation intermediates.

Compound specific isotope analysis (CSIA) is a promising technique which allows assessment of biodegradation through identification of the shift in the stable isotope ratio of elements present in the compound of interest (recent review by Hofstetter *et al.*, 2008). As biodegradation of a compound proceeds, enrichment of molecules containing ^{13}C and ^{2}H may occur due to the slightly increased strength of bonds involving these heavier isotopes. The extent of stable isotope enrichment depends on the kinetic isotope effect which is specific to the biodegradation mechanism and types of enzymes involved (Rosell *et al*., 2007). Thus, a characteristic isotopic enrichment factor (ε) can be determined for a specific compound and biodegradation process. Studies show that a multidimensional analysis of enrichment factors for multiple elements (i.e., carbon and hydrogen) can enable the identification and characterization of the predominant degradation pathway when there are multiple competing degradation processes (Gray *et al*., 2002; Zwank *et al*., 2005; Kuder *et al*., 2005; Rosell *et al*., 2007).

To use CSIA for quantitative measurements of *in situ* MTBE biodgradation, we need reliable compound-specific isotopic fractionation factors (ε) values for specific elements and an understanding of the factors that influence isotopic fractionation. In this study, carbon and hydrogen isotope fractionation were determined for both aerobic and anaerobic MTBE biodegradation. This is the first report of carbon and hydrogen isotopic enrichment factor values for MTBE degradation mediated by *Variovorax paradoxus*, a

psychrophilic aerobic bacterium. A bacterial consortium containing *V. paradoxus* was incubated under different temperature conditions to determine the effect of temperature on the isotopic enrichment factors.

We also determined εC and εH in three sets of anaerobic MTBE-degrading sediment enrichment cultures, representing two sediment collection sites, two electron accepting conditions and the presence or absence of syringic acid as a co-substrate. The results are an important addition to the growing body of data regarding factors that affect isotopic enrichment factors for anaerobic MTBE degradation.

III. Materials and Methods

A. Aerobic cultures

A cold-active MTBE-degrading mixed bacterial culture designated CL-EMC-1 was provided by Gennadi Zaitsev (Rovaniemi, Finland). The culture consists of the MTBE dgrading organism *Variovorax paradoxus* strain CL-8 and two other organisms, *Hyphomicrobium facilis* strain CL-2 and *Methylobacterium extorquens* strain CL-4. Cultures were grown in 1 L media bottles containing minimal salts CLM medium with ~1100 μM MTBE (Zaitsev *et al*., 2007).

B. Anaerobic enrichment cultures

Anaerobic sediment enrichment cultures from New York Harbor (NYH) and Arthur Kill Inlet (AK) between New York and New Jersey were previously established (Somsamak *et al*., 2001, 2005). Anaerobic cultures under methanogenic or sulfidogenic conditions were maintained at 28°C in glass serum vials capped with Teflon-coated

stoppers and aluminum crimp seals, using strict anaerobic technique. Cultures were repeatedly transferred, at 6-12 month intervals, into fresh medium and enriched with either MTBE (Aldrich, Milwakee, WI) alone as a carbon source or with MTBE and the methoxylated aromatic compound, syringic acid (Sigma, St Louis, MO), as a co-substrate (Youngster *et al*., 2008; Chapter 2). Select enrichment cultures, representing 10^{-8} transfers of the original sediments, were chosen for CSIA.

C. Experimental setup

Multiple batch experiments were set up with aerobic cultures containing *V. paradoxus* CL-8. Three different temperature conditions were used for growth; 10°C, 20°C and 28°C with 4-6 replicate 100 ml cultures incubated under each temperature condition. Cultures were initially spiked with MTBE to a final concentration of 1350 µM.

Six batch experiments were set up with anaerobic cultures. Two 100 ml cultures each were selected from 3 different enrichment conditions; AK sediment under sulfidogenic conditions with MTBE as a sole substrate (AKsulf), NYH sediment under methanogenic conditions with MTBE as a sole substrate (NYmeth) , and NYH sediment under methanogenic conditions with both MTBE and syringic acid as co-substrates (NYsyr). Cultures were spiked with anaerobic MTBE stock to a final concentration of ~1100 µM. NYsyr cultures were spiked with anaerobic syringic acid stock to a concentration of 50 µM.

Cultures consisting of media with MTBE were used as abiotic controls for both aerobic and anaerobic experiments. At each sampling timepoint, the MTBE

concentration was measured. Liquid samples of 1 ml were removed to 15 ml serum vials containing 0.6 g NaCl and stored at -20°C until isotope analysis.

D. Analytical methods

The concentration of MTBE was monitored regularly using gas chromatography with flame ionization detection (Somsamak *et al*., 2001). The concentration of syringic acid was monitored by high-performance liquid chromatography (Youngster *et al*., 2008; Chapter 2).

Stable isotope analyses were conducted at the Stable Isotope Laboratory of the UFZ Centre for Environmental Research in Leipzig-Halle, Germany. Stable carbon and hydrogen isotope compositions were determined using a gas chromatograph (6890 series; Agilent Technology) coupled with a combustion interface (ThermoFinnigan GC-combustion III; ThermoFinnigan, Bremen, Germany) to either a Finnigan MAT 252 (for carbon analysis) or 253 (for hydrogen analysis) isotope ratio mass spectrometer (ThermoFinnigan, Bremen, Germany). Each sample was analyzed by headspace injections conducted at least in triplicate.

In this method, carbon and hydrogen isotopic compositions are reported as δ values in parts per thousand (‰) relative to the international standards, Vienna Pee Dee Belemnite and Vienna Standard Mean Ocean Water, respectively. Isotopic fractionation factors (α) were calculated using these δ values with the Rayleigh equation:

$$\ln(R_t/R_o) = (1/\alpha - 1) \times \ln(C_t/C_o)$$

Where R is the isotope ratio and C is concentration. Isotopic enrichment factors (ε) were estimated by plotting $\ln(R_t/R_o)$ vs. $\ln(C_t/C_o)$ and determining the slope (b) of the resulting line by linear regression.

$b = (1/\alpha - 1)$ and $\varepsilon = 1000 \times b$.

IV. Results

A. C and H isotope fractionation during aerobic MTBE degradation

Carbon and hydrogen isotope fractionation was measured during MTBE degradation by the aerobic cold-active mixed bacterial consortium designated CL-EMC-1 (Zaitsev *et al*., 2007) which contains *Variovorax paradoxus* as the MTBE-degrading organism. The culture was incubated under three different temperature conditions; 10°C, 20°C and 28°C. Complete degradation of MTBE occurred most rapidly at 20 and 10°C, with slower degradation at 28°C (Figure 5.1.A), consistent with previous observations of degradation by this cold-active organism (Zaitsev *et al*., 2007). All CL-EMC-1 cultures showed some ^{13}C enrichment in the residual substrate pool during the degradation timecourse (Figure 5.1.B). The average Cε values at 10°C, 20°C and 28°C were near identical, at 1.1 to 1.2‰ (Table 5.1.), indicating that temperature conditions during growth of *V. paradoxus* on MTBE did not affect Cε. Enrichment of ^{2}H was also observed in every culture (Figure 5.1.C), with Hε values of -13.8‰, -13.2‰ and -18.0‰ at incubation temperatures of 10°C, 20°C and 28°C, respectively. This indicates that, like the Cε, the Hε is also not substantially influenced by temperature conditions during growth of *Variovrax paradoxus* on MTBE. The hydrogen isotope fractionation is

consistent with biodegradation involving breakage or formation of a C-H bond in the initial transformation reaction of MTBE.

B. C and H isotope fractionation during anaerobic MTBE degradation

Anoxic conditions predominate in fuel-contaminated aquifers, therefore anaerobic MTBE degradation is an essential component of remediation by natural attenuation. The anerobic cultures used in this study were sulfidogenic and methogenic enrichment cultures from New York-New Jersey Harbor estuary sediments maintained with either MTBE as the sole carbon source (NYmeth and AKsulf) or for the most recent two transfers with MTBE and syringate as a co-substrate (NYsyr). All enrichment cultures have sustained MTBE utilization over an enrichment period consisting of repeated transfer into fresh medium over several years for total dilutions of 10^{-8} of the original cultures.

The high concentration requirements for hydrogen CSIA necessitated that we used an MTBE concentration above 1000 μM, which is considerably higher than concentrations typically used for enrichment. After 210 days of incubation, between 20 to 31% MTBE depletion was observed in each culture (Figure 5.2.A). Similar degrees of ^{13}C enrichment were seen in each of the anaerobic MTBE degrading cultures (Figure 5.2.B). The average Cε for NYmeth, NYsyr and AKsulf cultures (two of each), respectively, were -7.04‰, -6.73‰ and -7.14‰ (Table 5.2.). Ratios of ^{2}H:^{1}H were not significantly changed during the course of MTBE degradation in NYmeth and AKsulf cultures, but a strong ^{2}H enrichment was observed in NYsyr cultures (Figure 5.2.C). The NYsyr cultures had an average Hε of -41.12‰ with high R^2 values supporting this data,

while R^2 values for Hε values determined for NYmeth and AKsulf cultures were all less than 0.100.

V. Discussion

Accurate monitoring of natural attenuation is important for assessment of biodegradation activity and potential in contaminated environments. It can also further our understanding of how microbial processes mediating bioremediation are influenced by various amendments and other engineering approaches. As a tool for quantifying biotransformation processes in the environment, CSIA overcomes many analytical challenges associated with biodegradation monitoring. When the compound of interest has a reliable stable isotope enrichment factor, CSIA can clearly distinguish between concentration changes due to physical processes versus degradation. In environmental samples, the influence of different microorganisms and different degradation mechanisms complicates the determination of isotopic fractionation factors. Multidimensional CSIA could help resolve this complication by enabling the identification of, not just biodegradation in the environment, but of specific biotransformation processes based on the relationships of isotopic fractionation factors for multiple elements. This technique requires determination of stable isotopic fractionation factors of several elements for different degradation processes (for reviews, see Hofstetter *et al*., 2008). Compound specific isotopic fractionation factors can be established through laboratory microcosm experiments, like the present study, which presents new information about the carbon and hydrogen isotopic fractionation occurring in both anaerobic and aerobic samples.

This is the first examination of the carbon and hydrogen isotope fractionation during aerobic MTBE degradation by *V. paradoxus* in the cold-active CL-EMC-1 consortium. The relationship between Cε and Hε values determined for *V. paradoxus* does not directly correspond to patterns determined for other aerobic MTBE degrading strains. *Aquincola tertiaricarbonis* strain L108 and *Rhodococcus ruber* strain IFP2001 displayed lower εC values of -0.48‰ and -0.28‰, respectively, and no significant ^{2}H enrichment in either strain (Rosell *et al*., 2007). In MTBE degradation by *A. tertiaricarbonis* strain L108, the initial monooxygenase reaction attacking the methyl group of MTBE is catalysed by an enzyme encoded by the *ethABCD* genes (Muller *et al*., 2008) and a similar monooxygenase has been detected in *R. ruber* strain IFP2001 (Chauvaux *et al*., 2001), suggesting that these organisms have similar O-demethylation methods. The common lack of ^{2}H enrichment in MTBE degradation by either species suggests that this O-demethylation step does not involve a fractionation-inducing manipulation of a hydrogen bond.

Methylibium petroleiphilum strain PM1 and *Methylibium* sp. strain R8 are two other aerobic MTBE degrading species for which carbon and hydrogen enrichment factors have been determined, with Cε and Hε of -2.0‰ to -2.4‰ and -33‰ to -37‰, respectively, for strain PM1 (Gray *et al*., 2002) and -2.4‰ (Cε) and -42‰ (Hε) for strain R8 (Rosell *et al*., 2007). These strains are phylogenetically similar and the carbon and hydrogen fractionations seen are almost identical, indicating that they likely degrade MTBE by a similar mechanism. That differences in carbon and hydrogen fractionation reveal differences in degradation between pathways is also supported by genomic data. The genome of *M. petroleiphilum* strain PM1 does not contain *ethABCD* genes (Kane *et*

al., 2001), so the initial O-demethylation of MTBE must be mediated by a different enzyme.

From the hydrogen isotope fractionation observed, it appears that the breakage or formation of a hydrogen bond is involved in the initial reaction in strains PM1 and R8, as well as in *V. paradoxus*. The average εC and εH values of -1.1‰ and -14.6‰, respectively, in the present study indicate that the degradation pathway used by *V. paradoxus* causes stronger fractionation (of both carbon and hydrogen) than in the L108- and IFP2001-type pathways, but weaker fractionation than in the PM1- and R8-type pathway. These findings lend support for the proposed pathway for aerobic MTBE degradation by *V. paradoxus*,which includes an initial hydroxylation of the O-methyl group of MTBE, with concomitant breakage of the methyl C-H bond, forming a *tert*-butoxymethanol intermediate (Zaitsev *et al*., 2007). The difference in the strength of the ^{2}H enrichment factor during degradation by *V. paradoxus* and the other two organisms may be due to the use of a different enzyme for the initial attack. This is a likely explanation, since *V. paradoxus* is a psychrophilic organism, capable of MTBE degradation at culture temperatures ranging from 3 to 30°C, with the highest rate of degradation between 10 and 22°C (Zaitsev *et al*., 2007).

V. paradoxus is a particularly interesting organism because it readily degrades MTBE at 10°C. The temperature of many contaminated water supplies is below the ambient 20-30°C that most MTBE degradation studies are conducted at. Therefore *V. paradoxus* may play an important role in bioremediation in cold groundwater enrichments. Aerobic MTBE degradation has already proven useful in *in situ* MTBE treatment and using bioreactors for aboveground water treatment (Wilson, 2003). CSIA

could be a valuable tool for assessing the effectiveness of bioremediation methods, both in process development and in the field. Analyses of stable isotope fractionation during MTBE degradation in pure bacterial cultures and mixed cultures containing known MTBE degrading organisms demonstrate that degradation by different strains produces different Cε and Hε values. Knowing the carbon and hydrogen isotope fractionation factors during the degradation process of *V. paradoxus* will enable the use of CSIA for detecting degradation by *V. paradoxus* in the environment and for evaluating the effectiveness of efforts to use the organism in MTBE-degrading bioreactors.

Carbon isotope fractionation due to anaerobic MTBE degradation is consistently much stronger than the fractionation seen during aerobic MTBE degradation. All microcosm and *in situ* CSIA studies have reported significant ^{13}C enrichment during anaerobic MTBE degradation (Kolhatkar *et al*., 2002; Kuder *et al*., 2005; Somsamak *et al*., 2005, 2006) and field studies have demonstrated the use of CSIA to identify anaerobic MTBE biodegradation at contaminated sites (Kolhatkar *et al*., 2002; Kuder *et al*., 2005; Zwank *et al*., 2005; McKelvie *et al*., 2007). εC values for anaerobic MTBE degradation span a wide range, from -19.7‰ to -4.2‰, with the averages falling between -15.6‰ and -7.0‰, indicating that there are likely different reaction mechanisms for anaerobic MTBE degradation. No anaerobic MTBE degrading organism has yet been isolated making it impossible to confirm whether or not different isotopic fractionation values correlate to degradation by different species. The present study is the first to describe anaerobic hydrogen enrichment factors that are substantially different between cultures, possibly providing CSIA evidence of multiple anaerobic MTBE degradation mechanisms.

It is not apparent why the cultures enriched on MTBE with syringic acid as a co-substrate show a strong hydrogen fractionation during MTBE degradation. Addition of syringic acid as a co-substrate can increase the rate of MTBE utilization (Youngster *et al*., 2008; Chapter 2), presumably by encouraging the growth of organisms that are capable of O-demethylating activity by providing them with a more easily O-demethylated substrate. One might speculate that if there are multiple MTBE-degrading species present in a single enrichment culture, syringate stimulates MTBE degradation by a species that metabolizes MTBE by a mechanism similar to that proposed for aerobic species, wherein C-H bond breakage is involved, producing ^{2}H enrichment.

The current study also finds that addition of syringate as a co-substrate does not influence the Cε of the degradation reaction. As in previous studies (Somsamak *et al*., 2005, 2006), Cε was not influenced by the redox condition of the enrichment culture. This correlates well with findings that the electron accepting condition is not directly linked to the MTBE-degradation process and that the O-demethylation reaction is likely mediated by acetogenic bacteria rather than sulfidogenic or methanogenic organisms (Chapter 2). In this study we observed a slightly smaller carbon isotopic enrichment factor than measured in previous studies of earlier generations of anaerobic MTBE-degrading enrichment cultures (Somsamak *et al*., 2005, 2006). One possible reason for this could be that the community changes with enrichment, selecting for one MTBE degradation mechanism or MTBE-degrading species over others if there are multiple mechanisms or MTBE-degrading species active in the community. The additional enrichment of the cultures between previous (Somsamak *et al*., 2005, 2006) and the current CSIA studies might account for community changes that result in a decreased Cε.

It is also possible that the increased initial concentration of MTBE used in the present study could have exercised some additional selective pressure on the community if some species were impacted by the higher concentrations of MTBE or TBA, which accumulated as degradation progressed.

Conclusions

The results of this study illustrate several ways in which CSIA of laboratory cultures can be useful beyond determination of stable isotope enrichment factors for use of CSIA *in situ*. Multidimensional CSIA provides novel information about the organisms and degradation processes that are occurring in a culture. The need for better understanding of uncultured microbes that are mediating important biodegradation processes requires development of improved techniques to study microbial processes independent of cultural isolation. Even when the organisms responsible for degradation can be isolated, CSIA of the degradation process can be useful for studying the degradation process. While several aerobic MTBE-degrading organisms have been isolated, they appear to use different enzymes and pathways for aerobic MTBE degradation and only a few of these enzymes have been identified. Multidimensional CSIA is a tool for differentiating between processes even without a genome sequence or specific enzyme to search for. Combining multidimensional CSIA with molecular community characterization to determine how changes in isotopic fractionation correlate with taxonomic community changes in enrichment cultures may strengthen both types of analysis and bring us closer to an understanding of how MTBE degradation occurs under anaerobic conditions.

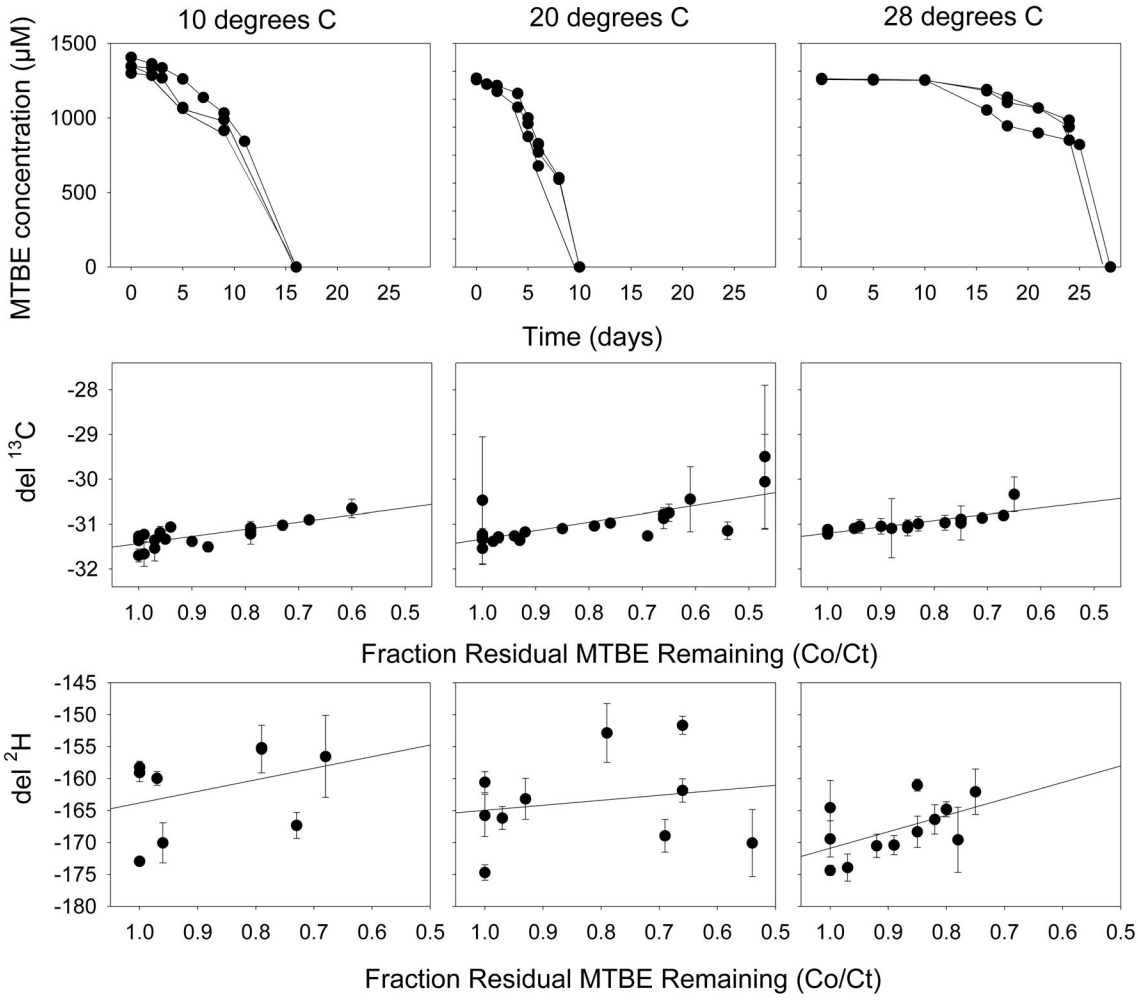


FIGURE 5.1. Depletion of MTBE (A) and corresponding carbon (B) and hydrogen (C) fractionation in aerobic MTBE-degrading cultures incubated at 10°C, 20°C and 28° C. Data from replicate cultures.

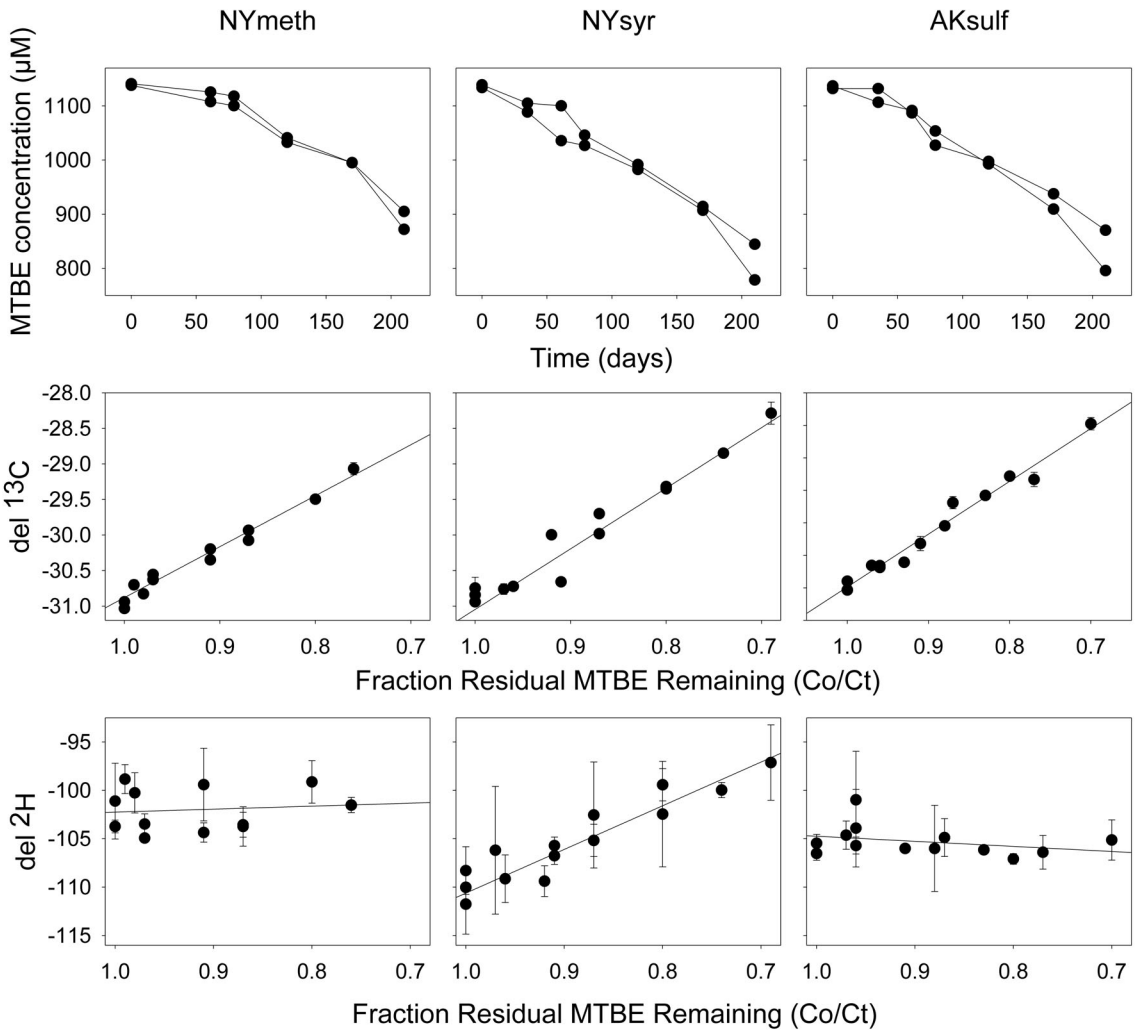


FIGURE 5.2. Depletion of MTBE (A) and corresponding carbon (B) and hydrogen (C) fractionation MTBE in anerobic MTBE-degrading cultures; NYmeth, NYsyr and AKsulf. Data from replicate cultures.

TABLE 5.1. Studies of stable isotope fractionation during aerobic MTBE biodegradation

Source	$^{13}C\varepsilon$ (‰) (95%CI)	$^{2}H\varepsilon$ (‰) (95%CI)	Reference
V. paradoxus 10°C (CL-EMC-1)	-1.15 (-1.23 to -1.07)	-13.75 (-15.13 to -12.37)	This study
V. paradoxus 20°C (CL-EMC-1)	-1.15 (-1.33 to -0.97)	-13.22 (-15.27 to -11.17)	This study
V. paradoxus 28°C (CL-EMC-1)	-1.13 (-1.21 to -1.09)	-17.95 (-18.98 to -16.92)	This study
V. paradoxus all temperatures (CL-EMC-1)	-1.13 (-1.26 to -1.00)	-14.58 (-15.99 to -13.17)	This study
Methylibium petroleiphilum strain PM1	-2.2 (-2 to -2.4)	-37 (-42 to -32)	Gray *et al*., 2002
Methylibium sp. strain R8	-2.4 (-2.5 to -2.3)	-42 (-46 to -38)	Rosell *et al*., 2007
Aquincola tertiaricarbonis strain L108	-0.48 (-0.53 to -0.43)	insignificant	Rosell *et al*., 2007
Rhodococcus ruber strain IFP2001	-0.28 (-0.34 to -0.22)	insignificant	Rosell *et al*., 2007
Aerobic microcosm, mixed	-1.65 (-1.5 to -1.8)	-47.5 (-66 to -29)	Gray *et al*., 2002
Groundwater from a contaminated field site in S. America	N/A	-3.3 (-3.7 to -2.9)	Zwank *et al*., 2005
Aerobic microcosms from aquifer sediments	-1.74 (-2.02 to -1.46)	N/A	Hunkeler *et al*., 2001
Field study, contaminated groundwater from Port Hueneme, CA	-1.4 (CI N/A)	N/A	Lesser *et al*., 2008

TABLE 5.2. Studies of stable isotope fractionation during anaerobic MTBE biodegradation.

Source	$^{13}C\varepsilon$ (‰) (95% CI)	$^{2}H\varepsilon$ (‰) (95% CI)	Reference
Anaerobic enrichment culture Arthur Kill Inlet Sediment (AKsulf)	-7.04 (-7.16 to -6.92)	-41.11 (-42.26 to -39.96)	This study
Anaerobic enrichment culture; New York Harbor sediment (NYsyr)	-6.73 (-6.90 to -6.56)	-4.30 (-6.14 to 2.46)	This study
Anaerobic enrichment culture; New York Harbor sediment (NYmeth)	-7.14 (-7.22 to -7.06)	4.01 (-6.07 to -1.95)	This study
Anaerobic enrichment culture from Arthur Kill or Coronado Cay sediment	-14.4 (-15.1 to -13.7)	N/A	Somsamak *et al*., 2005; 2006
Laboratory microcosms	-9.16 (-14.16 to -4.16)	N/A	Kolhatkar *et al*., 2002
Field experiment, groundwater samples, NJ gas station	-8.10 (-8.95 to -7.24)	N/A	Kolhatkar *et al*., 2002
Groundwater from a contaminated field site in S. America	N/A	-15.6 (-17.6 to -13.6)	Zwank *et al*., 2005
Microcosm enrichment culture, New Jersey contaminated gas station	-13 (-14.1 to -11.9)	-16 (-21 to -11)	Kuder *et al*., 2005

Chapter 6

Discussion

I. Discussion

In this study, we have elucidated crucial features of MTBE degradation, identified some of the microorganisms mediating or involved in this process and investigated the conditions that govern this capability. A combination of microbiological, molecular and geochemical analytical techniques were used (Table 6.1). Biodegradation of MTBE is a very desirable remediation method for contaminated groundwater because transformation of the pollutant actually eliminates the chemical from the environment rather than merely removing it from the water. Given the dearth of information about anaerobic MTBE degrading organisms and processes, the data uncovered about the anaerobic MTBE degrading sediment enrichment cultures here represents a significant contribution to the field. The inconsistency and rarity of anaerobic MTBE degradation activity in newly established enrichment cultures has been demonstrated by many studies that have observed degradation in only a few of many replicate cultures started from contaminated soils (Suflita and Mormile, 1993; Borden *et al*., 1997; Somsamak *et al*., 2001; Chen *et al*., 2005; Fischer *et* al., 2005; Martienssen *et al*., 2006). The availability of established, highly enriched cultures provided an opportunity to study the effects of various cultural amendments on anaerobic MTBE degradation in a more controlled setting than is usually possible. Table 6.1 describes the different cultures used in this study. In these cultures, we first tested the effects of gasoline and gasoline components because these compounds are likely to be present in polluted environments where we want anaerobic MTBE degradation to occur (Chapter 2). This is of importance, because the anaerobic MTBE degradation activity observed in these sediment enrichment cultures would immediately be much less appealing to study for practical *in situ* remediation if it was significantly

inhibited by the presence of gasoline. Fortunately, addition of methanol, benzene, toluene, ethanol or gasoline did not substantially hinder MTBE degradation.

The studies of substrate amendments with methoxylated aromatic compounds also produced promising results from the perspective of investigating anaerobic MTBE degradation for eventual development of *in situ* remediation (Chapter 2). We found that MTBE degradation rates were increased by addition of low concentrations of the lignoaromatic compounds; syringate and trimethoxybenzoate. These are naturally occurring, non-toxic compounds that could potentially be added to contaminated aquifers to stimulate or enhance anaerobic MTBE degradation *in situ*. Similar compounds, guaiacol and vanillate, also increased the rate of anaerobic MTBE degradation upon repeated spikings with both MTBE and the co-substrate. HPLC analysis revealed depletion of the methoxylated aromatic compounds and accumulation of O-demethylated transformation products. This indicated that the enrichment cultures were utilizing the methyl group of both MTBE and the methoxylated aromatic compounds. Since the first (and only) transformation step observed in our anaerobic MTBE degrading enrichment cultures is an O-demethylation reaction, resulting in TBA accumulation, it is likely that the MTBE-degrading organisms are O-demethylating the co-substrates by the same mechanism. Additionally, the methoxylated aromatic compounds used in this study (Figure 6.1.) are all known to be degradable by acetogenic bacteria, which use only the O-methyl group as a carbon source (Frazer, 1994), suggesting that the O-demethylation of MTBE may be mediated by acetogenic bacteria.

To further investigate the potentially acetogenic nature of the anaerobic MTBE degradation in the enrichment cultures, we used propyl iodide to determine whether or

not the O-demethylation reaction was likely dependent on corrinoid containing proteins. Many acetogenic and methanogenic O-demethylation pathways require a corrinoid-containing enzyme as a methyl acceptor (Stupperich and Kräutler, 1988; Kaufman *et al*., 1997; Naidu and Ragsdale, 2001) and propyl iodide binds corrinoids in a light-reversible manner, inhibiting corrinoid dependent reactions (Ghambeer *et al*., 1971; Choi, 1994). We observed light-reversible inhibition of MTBE degradation in the presence of propyl iodide, strongly suggesting that O-demethylation of MTBE in the anaerobic enrichments is corrinoid dependent. There is strong evidence that MTBE O-demethylation is not mediated by methanogens, from studies showing persistent MTBE degradation in the presence of the methanogenesis inhibitor, bromoethane sulfonate (Somsamak *et al*., 2001, 2006). This study also found a lack of MTBE degradation in the presence of the bacterial protein synthesis inhibitor, rifampicin (Chapter 2). From this, it can be inferred that the anaerobic O-demethylation of MTBE may be mediated by acetogenic bacteria. We have proposed a possible pathway showing how acetogens could obtain energy from the O-demethylation of MTBE (Figure 6.2.).

The strong evidence indicating that the anaerobic MTBE-degradation reaction is mediated by bacteria, not archaea, permitted us to focus molecular characterization studies exclusively on bacterial SSU rRNA (Chapter 3). Anaerobic enrichment culture populations were characterized by T-RFLP analysis of their 16S rRNA genes (Chapter 3). Comparison of T-RFLP profiles from enrichment cultures established from different sediments revealed great divergence in the community population based on site location. Profiles of communities from different locations displayed substantially different profiles with few major T-RF peaks in common. When fingerprints of enrichments from different

sediments shared a T-RF, it was rarely shared at a similar intensity, often being a major peak in some communities and a very minor peak in others. The presence of multiple T-RF peaks in the community fingerprints of cultures that had been enriched for several years and up to seven transfers, indicates that several different phylotypes are still present in these microcosms (Chapter 3 and 4). This suggests that cross-feeding of carbon is common in the population. There may also be multiple MTBE-degrading organisms in each culture, however, given the rarity of anaerobic MTBE degradation activity in the environment, it is unlikely that all phylotypes present are capable of MTBE O-demethylation. It is possible that syntrophic inter-species interactions of a consortium are required for appreciable anaerobic MTBE degradation to occur, which would explain the difficulty of isolating an organism with MTBE degrading capability. If, as suggested, acetogenesis is coupled to the anaerobic O-demethylation of MTBE, perhaps O-demethylation of MTBE is dependent on the presence of other species that can remove the acetate. Such a relationship has been observed between methanogens and acetogens by Conrad *et al.* (1985).

Comparative T-RFLP analysis of one enrichment culture, following sequential transfers with MTBE as a sole carbon source, revealed that the community complexity decreased and only three dominant phylotypes were maintained or increased over time (Chapter 3). To identify the bacteria associated with different T-RFs, a clone library of 16S rRNA gene sequences was constructed and sequenced. All the predominant T-RFs observed in T-RFLP analysis of the community were identified from the clone library. Phylogenetic analysis compared clones to the 16S rRNA gene sequences of the closest cultured organisms and several known O-demethylating bacteria. The tree illustrated that

the clones span several phyla and that the three dominant phylotypes clustered with the phyla *Deltaproteobacteria*, *Chloroflexi* and *Firmicutes* (Chapter 3, Figure 3.4.). Any of these phyla could likely contain an organism with acetogenic O-demethylating potential. The *Deltaproteobacteria* are well known for fermentation of different substrates. Members of the *Firmicutes* include several organisms known to O-demethylate aryl-methyl ether compounds, like those transformed by anaerobic MTBE degrading enrichment cultures. The partial 16S rRNA sequence of the *Firmicutes* clone in this study showed only 77% to 79% similarity to acetogens with known O-demethylation activity: *Acetobacterium woodii, Eubacterium limosum,* and *Syntrophococcus sucromutans* (Frazer, 1994). The clone which appears to be most heavily selected for by enrichment of MTBE clustered with the *Chloroflexi*, a metabolically diverse group with representatives widely distributed in the environment and found in many enrichment culture studies.

We were unable to determine, based on phylogeny, which organism was responsible for MTBE O-demethylation in this anaerobic enrichment culture. It is likely that there may be several bacteria capable of MTBE O-demethylation. To link functional activity within the community to phylogenetic characterization, we conducted the first stable isotope probing experiments with anaerobic MTBE degrading enrichment cultures (Chapter 4). Three DNA samples were analyzed from anaerobic MTBE degrading enrichments that had been fed ^{13}C labeled MTBE as the growth substrate. Two of these samples were from the same New York Harbor sediment enrichment culture (NYH1) at different timepoints early in degradation. The third sample was from a second New York Harbor sediment enrichment culture (NYH2). In the NYH2 sample and the earlier

timepoint of the NYH1 sample, comparative T-RFLP analysis of PCR amplified 16S rRNA genes of separated ^{12}C and ^{13}C labeled DNA revealed the presence of fewer T-RFs from the ^{13}C labeled DNA than in the total ^{12}C DNA profile. In the NYH1 culture, where there was also a later timepoint available for comparison, it was apparent that over time the ^{13}C label was incorporated by all members of the enrichment culture. This indicates that some members of the community appreciably assimilate ^{13}C from MTBE into their DNA before others. One or more of the organisms represented by peaks in the ^{13}C community profile are likely responsible for the initial O-demethylation of MTBE in these cultures. A major T-RF of 168 bp was present in each community analyzed, including all ^{13}C labeled communities from cultures NYH1 and NYH2, suggesting that this organism is central to the MTBE degradation process in this community and possibly responsible for MTBE O-demethylation. The eventual distribution of the ^{13}C label throughout the community population in the NYH1 culture supports previous suggestions that substantial cross-feeding takes place in the enrichment cultures. Utilization of carbon from decaying O-demethlyators allows multiple species to be sustained even when only one or a few can utilize carbon from the O-methyl group of MTBE.

Follow-up experiments to this study will involve constructing and sequencing a clone library of the NYH1 community used for SIP analysis to determine the phylogeny of the organisms that are principally ^{13}C labeled during ^{13}C-MTBE degradation. It will also be interesting to compare the sequences of clones from this community to sequences of clones from the Arthur Kill enrichment culture and find out if the clones of interest in two different cultures bear any phylogenetic similarity to each other. Results of this

initial SIP study indicated that DNA-SIP is an effective method for studying carbon flow in anaerobic MTBE degrading enrichment cultures. We confirmed that isotopic incorporation of ^{13}C from the labeled MTBE is sufficient for detection of ^{13}C labeled DNA in extracted samples. Based on the results of this study, recommendations can be made for experimental design adjustments for future SIP studies which may allow analysis of a greater number of timepoint samples from each culture. This should provide further details about the carbon flow in the enrichment culture communities and more opportunities to identify the earliest catabolizers of ^{13}C following ^{13}C-MTBE O-demethylation.

Identification of microorganisms responsible for MTBE biodegradation will provide molecular bioindicators for monitoring biodegradation *in situ* and evaluating the effectiveness of assisted bioremediation technologies. However, the ability to detect the presence of MTBE degrading organisms in contaminated groundwater will not necessarily tell us whether or not these organisms are actively degrading MTBE. In a dynamic system, concentration measurements are also insufficient for this purpose. Compound specific isotope analysis (CSIA) is a method of monitoring biodegradation in contaminated environments which is independent of molecular characterization of the community and overcomes several analytical challenges associated with monitoring microbiological processes *in situ*. CSIA has been used to detect both anaerobic and aerobic MTBE degradation in field studies using stable isotope enrichment factors determined in laboratory studies (Hunkeler *et al*., 2001; Gray *et al*., 2002; Kolhatkar *et al*., 2002; Kuder *et al*., 2005; Zwank *et al*., 2005; Somsamak *et al*., 2005, 2006; Rosell *et al*., 2007; Lesser *et al*., 2008). More complete information about the stable isotope

enrichment factors for fractionation of multiple elements in a variety of aerobic and anaerobic cultures is necessary for multidimensional CSIA to be reliably used to detect MTBE biodegradation under environmental conditions. The present study determined carbon and hydrogen isotope enrichment factors for MTBE degradation in anaerobic MTBE degrading enrichment cultures, as well as cold-active aerobic MTBE degrading cultures (Chapter 5).

Cultures selected for the anaerobic CSIA studies were sulfidogenic and methanogenic enrichment cultures from New York-New Jersey Harbor estuary sediments (Arthur Kill Inlet and New York Harbor). These cultures had been maintained with either MTBE as the sole carbon source or, for the two most recent transfers, with MTBE plus the co-substrate, syringic acid. All cultures were found to have similar carbon isotope fractionation, with the average Cε for all communities estimated to be -7.0 ± 0.2‰ (Chapter 5). This value was substantially lower than those determined in previous studies of enrichment cultures from the same sediments, but not out of the range of Cε values reported for anaerobic MTBE degradation (-19.7‰ to -4.2‰) (Table 5.2.). One possible reason for the weaker carbon isotope fractionation observed in this study is that community composition changes with extensive enrichment on MTBE, as illustrated by T-RFLP analyses (Chapter 3). The increased concentration of MTBE used in the CSIA study, and subsequent higher amount of accumulated TBA, may also have exerted a selective pressure on the community during MTBE degradation, possibly by slowing the growth of a portion of the population that is sensitive to higher concentrations of TBA. The enrichment of stable isotopes is a result of kinetic isotope effects arising from the biodegradation mechanisms dependent on the particular enzymes involved (Rosell *et al*.,

2007). Thus, the measured isotope enrichment is an average of mechanisms that are operating to degrade the MTBE and will be a function of all factors contributing to MTBE degradation in the complex microbial community. A shift in population in response to perturbations such as TBA accumulation would be expected to alter the contributions of different degradation mechanisms, changing isotopic enrichment values.

The Hε values were insignificant in cultures where MTBE was the only substrate provided, however we observed a strong ^{2}H enrichment in cultures which included syringic acid as a co-substrate (Chapter 5). It is not clear why addition of syringic acid would have such an effect on the H fractionation during MTBE degradation. It is possible that syringic acid strongly encourages growth of MTBE degradation organisms that use a different O-demethylation mechanism than that used by the MTBE degrading organisms active in the culture when MTBE is the only carbon source present.

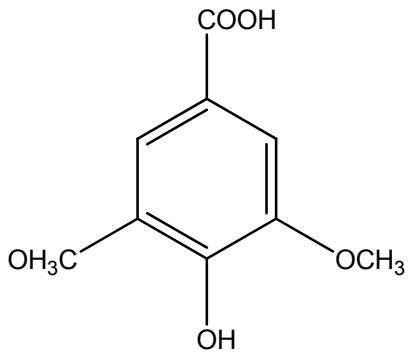
This study also includes analysis of carbon and hydrogen isotope fractionation during aerobic MTBE degradation. *V. paradoxus* readily degrades MTBE at 20 and 10°C, with slower degradation at 28°C, consistent with the psychrophilic nature of the organism. This bacterium, in the CL-EMC-1 consortium (Zaitsev *et al*., 2007), was incubated under these three different temperature conditions to determine the effect of temperature on enrichment of ^{13}C and ^{2}H during MTBE degradation. Average Cε and Hε values did not substantially differ with temperature , with average values of -1.1 ± 0.2‰ and -14.6 ± 2.1‰, respectively, indicating that, while the rate of degradation differed, the mechanism of degradation was consistent at all temperatures. The measurable enrichment of ^{2}H during aerobic MTBE degradation by *V. paradoxus* indicates that breakage or formation of an H bond is involved in the initial O-demethylation of MTBE.

This supports the proposed pathway which involves initial hydroxylation of the O-methyl group and breakage of the methyl C-H bond (Zaitsev *et al*., 2007). The significance of multidimensional CSIA is that it can be used to distinguish between different types of degradation pathways. Many different degradation pathways have the same initial substrate and end products, but result in different patterns of stable isotope fractionation, particularly when enrichment factors are compared for multiple elements. In this study of *V. paradoxus*, the relationship between Cε and Hε values did not directly match patterns determined for other aerobic MTBE degrading bacteria. This may indicate that *V. paradoxus* degrades MTBE via a different mechanism or using different enzymes than the other organisms for which C and H fractionation data is available (Table 5.1.). This is perhaps not an unexpected finding, given that other MTBE degrading strains are active at temperatures between 20 and 30°C. The psychrophilic nature of *V. paradoxus* makes it a potentially interesting organism for assisted bioremediation technologies for use in cold environments. Knowledge of C and H fractionation values for this MTBE degradation process will enable the use of CSIA for evaluating future assisted bioremediation studies.

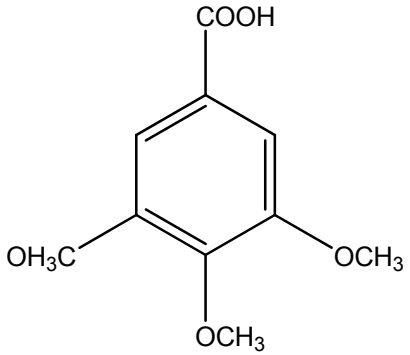
II. Conclusions

Overall, this study makes use of microbiological, molecular and geochemical analytical techniques to probe MTBE degrading cultures for information relevant to the enhancement and monitoring of MTBE biodegradation in contaminated groundwater. We identified O-methoxylated phenolic compounds as amendments that increase the MTBE biodegradation rate and could be applied *in situ* for assisted natural attenuation. Our experiments show that anaerobic MTBE O-demethylation is mediated by a corrinoid-dependent enzyme and is likely coupled to acetogenesis. The predominant phylotypes in enriched anaerobic MTBE degrading cultures were not closely related to previously characterized organisms and we provide the first experimental evidence that anaerobic microorganisms use O-methyl carbon from MTBE as a growth substrate for cell division. Our multidimensional CSIA results are the first indication of multiple mechanisms for anaerobic MTBE O-demethylation and corroborate the theorized pathway for aerobic MTBE degradation by the psychrophilic bacterium *V. paradoxus*. These advances in understanding MTBE degradation will support improvements in current bioremediation efforts and, moreover, guide future exploration of MTBE degradation processes

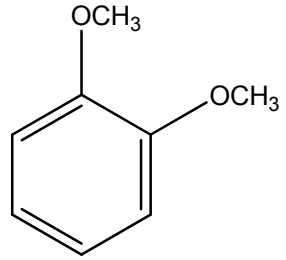
Figure 6.1. Structures of O-methoxylated phenolic compounds used in this study.



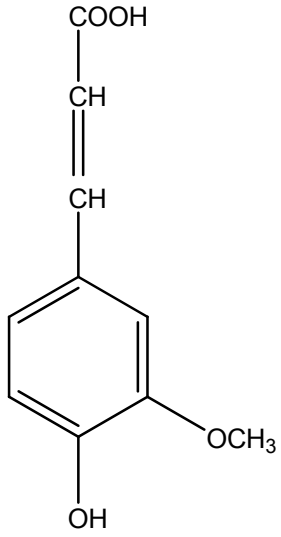
Syringic acid



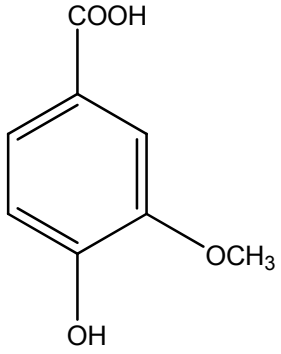
Trimethoxybenzoic acid



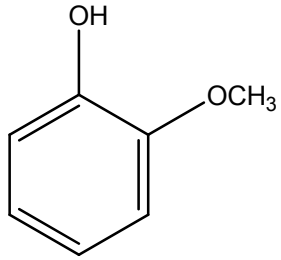
Veratrol



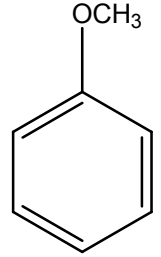
Ferulic acid



Vanillic acid



Guaiacol



Anisole

Figure 6.2. Proposed pathway for acetogenic metabolism of MTBE.

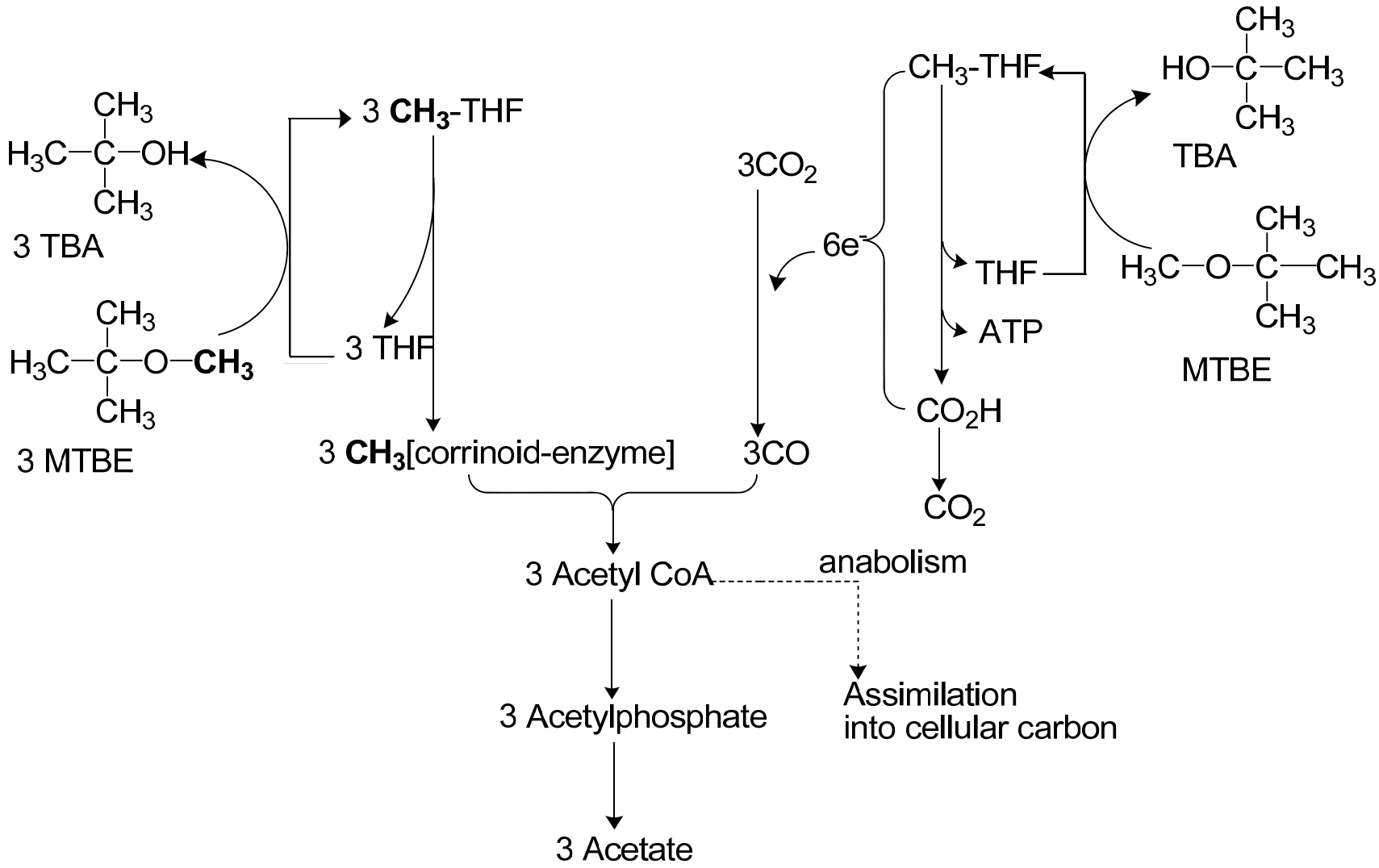


Table 6.1. List of anaerobic MTBE degrading cultures used.

Original culture	dilution	Experiment (reference)	Treatment
Arthur Kill Inlet (1) sulfidogenic	10^{-4}	Co-contaminants (Table 2.1)	100 ml 10^{-3} dilution, subdivided into fifteen 10 ml cultures and three 50 ml cultures at a 1:10 dilution
Arthur Kill Inlet (2) Sulfidogenic	10^{-4}	Multiple methoxy-aromatic compounds (Fig. 2.2, 2.3; Table 2.2)	50 ml 10^{-3} dilution, subdivided into twenty-four 10 ml cultures at a 1:10 dilution
	10^{-8}	CSIA (Fig. 5.2)	Portion of Arthur Kill Inlet (2) not used for methoxy-aromatic experiments eventually yielded two 100 ml cultures at a 10^{-8} dilution of the original
Arthur Kill Inlet methanogenic	10^{-3}	T-RFLP (Fig. 3.2, 3.5)	
	10^{-5}	T-RFLP (Fig. 3.2)	
	10^{-7}	Clone/sequence, T-RFLP (Fig. 3.1, 3.2, 3.3, 3.4)	
New York Harbor; sulfidogenic	10^{-3}	Rifampicin (Fig. 2.5)	25 ml 10^{-2} dilution, subdivided into six 10 ml cultures at a 1:10 dilution
	10^{-5}	T-RFLP (Fig. 3.6)	Portion of the sulfidogenic NYH culture not used for rifampicin experiments eventually yielded a 50 ml culture at a 10^{-5} dilution of the original

Table 6.1. (continued)

Original culture	dilution	Experiment (reference)	Treatment
New York Harbor (1) methanogenic	10^{-3}	Rifampicin (Fig. 2.5)	25 ml 10^{-2} dilution, subdivided into six 10 ml cultures at a 1:10 dilution
	10^{-5}	T-RFLP (Fig. 3.6)	Portion of New York Harbor (1) not used for rifampicin experiments eventually yielded a 50 ml culture at a 10^{-5} dilution of the original
New York Harbor (2) methanogenic	10^{-4}	Propyl Iodide (Fig. 2.4)	50 ml 10^{-3} dilution, subdivided into fifteen 10 ml cultures at a 1:10 dilution
(2-1)	10^{-7}	T-RFLP (SIP) (Fig. 4.1, 4.2)	Portion of New York Harbor (2) not used for propyl iodide experiments eventually yielded a 100 ml culture at a 10^{-7} dilution of the original. Separated from culture 2-2 since 10^{-4}.
(2-2)	10^{-7}	T-RFLP (SIP) (Fig. 4.3)	Portion of New York Harbor (2) not used for propyl iodide experiments eventually yielded a 100 ml culture at a 10^{-7} dilution of the original. Separated from culture 2-1 since 10^{-4}.
New York Harbor (3) methanogenic	10^{-3}	T-RFLP (Fig. 3.5)	
(3-1)	10^{-8}	CSIA (Fig. 5.2)	Separated from culture 3-2 since 10^{-3}.
(3-2)	10^{-8}	CSIA (Fig. 5.2)	Separated from culture 3-1 since 10^{-3}. Grown on MTBE and syringic acid since 10^{-7}
Graving Dock; sulfidogenic	10^{-3}	T-RFLP (Fig. 3.6)	
Graving Dock; methanogenic	10^{-3}	T-RFLP (Fig. 3.5, 3.6)	

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